REMARKS

This Response is being filed in connection with the Office Action mailed May 21, 2004. Claims 7 to 10, 13 to 16, 19, 20, 27 to 29, 31 to 33, 35 and 36 are pending. Claims 13 to 16, 28, 32, 35 and 36 stand withdrawn from consideration as directed to a non-elected invention. New claims 37 to 42 are submitted herewith. Accordingly, upon entry of the Response, claims 7 to 10, 19, 20, 27, 29, 31, 33 and 37 to 42 are under consideration.

Regarding the New Claims

New claims 37 to 42 are supported throughout the specification. In particular, claims 37 to 42, which depend from claims 7, 19, 27, 29, 31 and 33, respectively, are supported, for example, at page 4, lines 30-31, which discloses that the multivalent recombinant antibody can have an apparent affinity constant "of no less than 10^{10} M^{-1}." Thus, as new claims 37 to 42 are supported by the specification, no new matter has been added and entry thereof is respectfully requested.

Regarding the Amendments

The amendment to claims 7, 19, 27, 29, 31 and 33 is supported throughout the specification. In particular, the amendment to recite that the antibody has an apparent affinity constant for ICAM-1 of no less than "10^{9} M^{-1}" is supported, for example, at page 4, lines 28-30, which discloses that the multivalent recombinant antibody can have an apparent affinity constant "of no less than 10^{9} M^{-1}." The amendment to recite that the "antibody comprises three or more antigen binding domains for ICAM-1," is supported, for example, by claim 3, as originally filed, and at page 2, lines 24-25, which discloses that the multimeric configuration of the multivalent recombinant antibodies includes "trivalent" antibodies. The amendment to recite that the "antibody is polymerized through a coiled-coil sequence," is supported, for example, by claims 4 and 5, as originally filed, and at page 2, line 29, to page 3, line 14, which discloses, *inter alia*, that a multivalent antibody can be polymerized through a polymerization domain, and that examples of polymerization domains include "coiled coil domains." (see, also, page 3, line 29, to page 4, line 14) Thus, as the amendment to claims 7, 19, 27, 29, 31 and 33 is supported by the specification, no new matter has been added and entry thereof is respectfully requested.

Regarding the Claim Objections

The specification stands objected to due to an alleged failure to comply with the requirements for sequences. Allegedly, SEQ ID NOs have not been appended to the specific sequences in the specification and claims.

Applicants submit that this objection is erroneous. In particular, none of the claims recite sequences which require appending sequence identifiers (i.e., SEQ ID NOs). As to the specification, Applicants filed an Amendment on August 13, 2003, to insert sequence identifiers in the specification where appropriate. In particular, the specification was amended to inserted SEQ ID NOs on pages 4, 12, 13 and 18. For the Examiner's convenience, a copy of the Amendment, as-filed on August 13, 2003, is submitted herewith as Exhibit 1. Accordingly, in view of the Amendment, the specification complies with the requirements for sequences under 37 C.F.R. §1.821(d), and Applicants respectfully request withdrawal of the objection to the specification.

I. REJECTION UNDER 35 U.S.C. §103(a)

The rejection of claims 7 to 9, 19, 20, 27, 29, 31 and 33 under 35 U.S.C. §103(a) as allegedly unpatentable over Adair *et al.* (WO 91/16927) in view of King *et al.* (U.S. Patent No. 6,307,026 B1) and Hodits *et al.* (J. Biol. Chem. 270:24078 (1995)) is respectfully traversed. Adair *et al.* purportedly describe "humanized CDR-grafted antibody for the binding of ICAM-1," which have the "same specificity as the R6-5-D5 antibody," recombinant antibodies "having two antigen binding sites," for treatment of "rhinoviral infection." Absent evidence to the contrary, the antibodies are presumed by the Patent Office to "bind ICAM-1 with a specificity of 10^8 M^{-1}." The Patent Office acknowledges that Adair *et al.* fail to describe "multivalent recombinant antibody having more than 2 antigen binding sites." Purportedly, King *et al.* describe "multivalent antigen binding proteins using various crosslinking agents." Hodits *et al.* purportedly describe single chain fragment antibodies against LDL receptor, and allegedly making single chain antibodies multivalent "using a myc-sequence tag." Allegedly, one skilled in the art would have been motivated "to utilize the recombinant antibodies taught by Adair *et al.* and to increase there binding capacity by crosslinking the antibodies." Allegedly, Hodits *et al.* motivate "a combination of ICAM-1 directed antibodies and LDL antibodies into a single use

formulation" to "gain entry into the host cell via the LDL receptor (minor group) and via the ICAM-1 (major group)."

In order to establish obviousness under 35 U.S.C. §103(a), there must have been, at the time of the invention, 1) a suggestion or motivation to modify the reference or combine references; 2) a reasonable expectation of success of producing the claimed invention; and 3) the reference or combined references must teach or suggest each and every claim limitation. Both the teaching or suggestion to make the claimed combination *and* the reasonable expectation of success must both be found in the prior art, not in Applicants' disclosure. See, e.g., *In re Vaeck*, 947 F.2d 488 (Fed. Cir. 1991) and *In re O'Farrell*, 853 F.2d 894, 903-904 (Fed. Cir. 1988).

Here, originally filed claims 7 to 9, 19, 20, 27, 29, 31 and 33 would not have been obvious over Adair *et al.* (WO 91/16927), King *et al.* (U.S. Patent No. 6,307,026 B1) or Hodits *et al.* (J. Biol. Chem. 270:24078 (1995)) alone, or in any combination, at the time of the invention. For example, the antibody described by Adair *et al.*, denoted as R6-5-D5, has a K_D of 7.7 10^8 M^{-1} for ICAM-1, which is not less than 10^8 M^{-1}, as is required by originally filed claims 7 to 9, 19, 20, 27, 29, 31 and 33 under 35. Submitted herewith as Exhibit 2 (Casasnovas *et al.*, J. Biol. Chem. 270:13216 (1995)) is a publication indicating that R6-5-D5 (denoted as R6.5) has a K_D of 7.7 10^8 M^{-1} for ICAM-1 (Exhibit 2, page 13221, Table II). Also submitted herewith as Exhibit 3 (Casasnovas *et al.*, J. Virol. 72:6244 (1998)) is a publication indicating that R6.5, at best, has a K_D of 1.6 10^8 M^{-1} for ICAM-1 subdomain 2 (Exhibit 3, page 6246, Table 3, 2D/199), which is also not less than 10^8 M^{-1}. Thus, Adair *et al.* (WO 91/16927) fail to describe an antibody having the recited affinity of originally filed claims 7 to 9, 19, 20, 27, 29, 31 and 33.

As to King *et al.* allegedly describing cross-linking reagents to increase binding capacity, there is no evidence to indicate that even if the antibodies to ICAM-1 were cross-linked using the reagents described by King *et al.* that the cross-linked antibodies would have increased antigen binding capacity. In this regard, in order for cross-linking agents to produce mutivalent antibodies having increased antigen affinity, the cross-linking must maintain the antigen binding domains of the antibody in a geometry that is compatible with antigen binding. Without a geometry compatible with antigen binding, multivalency will not increase antigen affinity. For example, if the antigen binding domains are in a side-by-side configuration, there will likely be steric hindrance that inhibits binding of one or both of the antigen binding domains. However, King *et al.* fails to teach how to cross link antibodies while maintaining a geometry compatible

with antigen binding. Thus, in the absence of such a teaching, the skilled artisan would not have had a reasonable expectation that multivalent antibodies produced using cross-linking agents would have increased antigen affinity. Furthermore, King *et al.* fail to teach or suggest protein polymerization domains.

Hodits *et al.* describe a myc sequence tag linked to an scFv that binds to LDL receptor. Hodits *et al.*, without showing any data, report stronger protection in the presence of anti-myc antibody, which the authors attribute to rendering the scFV bivalent. However, the purportedly stronger protection may be due to any number of reasons. For example, one alternative is that the anti-myc antibody binds to the myc-tagged scFv and provides greater steric hindrance of rhinovirus binding to LDL receptor. Another alternative is that the anti-myc antibody protects that myc-tagged scFv from degradation thereby providing stronger protection. Yet another alternative is that the antibody binding to the myc-tagged scFv may increase recycling of the LDL receptor from the cell surface so that less LDL receptor is available for virus binding. In this regard, unlike ICAM-1, LDL receptor is known to recycle rapidly from the cell surface. Hodits *et al.* provide no definitive evidence that the anti-myc tag binds to two scFvs to render them bivalent. Moreover, Hodits *et al.* did not report using any controls to distinguish among the various alternative reasons for the increased protection. Consequently, it is at most speculation to attribute the observed protection observed by Hodits *et al.* to multivalency.

In view of the foregoing, the cited references either fail to teach or suggest each and every element of originally filed claims 7 to 9, 19, 20, 27, 29, 31 and 33, or fail to provide a reasonable expectation of success of producing originally filed claims 7 to 9, 19, 20, 27, 29, 31 and 33. Consequently, the rejection of the originally filed claims under 35 U.S.C. §103(a) is improper.

In any case, solely in order to further prosecution of the application and without acquiescing to the propriety of the rejection, the claims have been amended as set forth above. The rejection will therefore be addressed as it may pertain to the amended and new claims.

Amended and new claims 7 to 10, 19, 20, 27, 29, 31, 33 and 37 to 42 recite that the multivalent antibody against ICAM-1 has an apparent affinity constant for ICAM-1 of no less than 10^9 M^{-1}, comprises three or more antigen binding domains for ICAM-1, and is polymerized through a coiled-coil sequence. Neither Adair *et al.*, King *et al.* nor Hodits *et al.* alone, or in any combination, teach or suggest such multivalent antibody against ICAM-1. Furthermore, neither

Adair *et al.*, King *et al.* nor Hodits *et al.* alone, or in combination, provide a reasonable expectation that such multivalent antibody against ICAM-1 could be produced at the time of the invention. Consequently, claims 7 to 10, 19, 20, 27, 29, 31, 33 and 37 to 42 would not have been obvious in view of Adair *et al.* (WO 91/16927), King *et al.* (U.S. Patent No. 6,307,026 B1) and Hodits *et al.* (J. Biol. Chem. 270:24078 (1995)). Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. §103(a) be withdrawn.

CONCLUSION

In summary, for the reasons set forth herein, Applicant maintains that claims 7 to 10, 19, 20, 27, 29, 31, 33 and 37 to 42 clearly and patentably define the invention, respectfully request that the Examiner reconsider the various grounds set forth in the Office Action, and respectfully request the allowance of the claims which are now pending.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's representative can be reached at (858) 509-4065.

Please charge any additional fees, or make any credits, to Deposit Account No. 50-2212.

Respectfully submitted,

Pillsbury Winthrop LLP

Date: 11.22.04

By _____

Robert M. Bedgood, Ph.D.
Reg. No. 43,488

Tel. No. (858) 509-4065
Fax No. (858) 509-4010

11682 El Camino Real
Suite 200
San Diego, CA 92130-2092
(619) 234-5000

Patent Appln. No. 09/555,446 PW File No. 014357-0278749 By: JRW/SLS
In the Matter of the Application of: FANG, FANG
Title: MULTIVALENT RECOMBINANT ANTIBODIES FOR...
Date Mailed: 8/18/2003 Due Date:

The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:

- [X] Transmittal letter
- [ ] Patent Application _____
- [ ] ___ pgs. Specification including ___ Claims, Abstract
- [ ] Drawings ___ sheets ___ formal ___ informal
- [ ] Oath/Declaration
- [ ] Assignment w/form PTO-1595
- [ ] Power of Attorney
- [ ] Small Entity Statement
- [ ] Deposit Account Authorization (in duplicate)
- [ ] Information Disclosure Statement, PTO-1449 w/___ refs.
- [ ] Check $ _____
- [X] Amendment/Response
- [ ] Request for Extension of Time (___ months) (patent)
- [ ] Terminal Disclaimer
- [ ] Notice of Appeal
- [ ] Appeal Brief (in triplicate)
- [ ] Letter to Official Draftsperson

- [ ] Request For _____
- [ ] Status Inquiry
- [ ] Certificate of Mailing
- [ ] Express Mail Certificate No. _____
- [ ] Certificate under § 3.73(b)
- [ ] Certificate of Correction (PTO Form 1050)
- [ ] Issue Fee Transmittal
- [ ] Fee Transmittal
- [X] Petition for REVIVAL of APPLICATION
- [X] SEQUENCE LISTING
- [X] Other: DISKETTE
- [X] SEQUENCE LISTING
- [ ] TRANSMITTAL
- [X] SEQUENCE LISTING
- [ ] STATEMENT
- [X] MARKED-UP PAGES
- [X] COPY of NOTICE TO COMPLY
- [ ] RESPONSE TO COMMUNICATION
- REGARDING POWER of ATTORNEY

EL989436417US

Patent Appln. No. 09/555,446 PW File No. 014357-0278749 By: JRW/SLS
In the Matter of the Application of: FANG, FANG
Title: MULTIVALENT RECOMBINANT ANTIBODIES FOR...
Date Mailed: 8/18/2003 Due Date:
The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:
[X] Transmittal letter
[ ] Patent Application _____
[ ] ___ pgs. Specification including ___ Claims/Abstract
[ ] Drawings ___ sheets ___ formal ___ informal
[ ] Oath/Declaration
[ ] Assignment w/form PTO-1595
[ ] Power of Attorney
[ ] Small Entity Statement
[ ] Deposit Account Authorization (in duplicate)
[ ] Information Disclosure Statement, PTO-1449 w/___ refs.
[ ] Check $ _____
[X] Amendment/Response
[ ] Request for Extension of Time (___ months) (patent)
[ ] Terminal Disclaimer
[ ] Notice of Appeal
[ ] Appeal Brief (in triplicate)
[ ] Letter to Official Draftsperson
[ ] Request For _____
[ ] Status Inquiry
[ ] Certificate of Mailing
[ ] Express Mail Certificate No. _____
[ ] Certificate under § 3.73(b)
[ ] Certificate of Correction (PTO Form 1050)
[ ] Issue Fee Transmittal
[ ] Fee Transmittal
[X] Petition for REVIVAL of APPLICATION
[X] SEQUENCE LISTING
[X] Other: DISKETTE
[X] SEQUENCE LISTING
[ ] TRANSMITTAL
[X] SEQUENCE LISTING
[ ] STATEMENT
[X] MARKED-UP PAGES
[X] COPY of NOTICE TO COMPLY
[ ] RESPONSE TO COMMUNICATION
REGARDING POWER of ATTORNEY
EL989436417US

Exhibit 1

FLAT RATE

Recycled Paper

EXPRESS MAIL
UNITED STATES POSTAL SERVICE®

www.usps.com

EXPRESS MAIL
POSTAGE REQUIRED
DOMESTIC USE ONLY

HO

EL 989436417 US

Mailing Label
Label 11-F June 2002

EXPRESS MAIL
UNITED STATES POSTAL SERVICE®

Post Office To Addressee

ORIGIN (POSTAL USE ONLY)

PO ZIP Code	Day of Delivery ☐ Next ☐ Second	Flat Rate Envelope ☐
Date In Mo. Day Year	☐ 12 Noon ☐ 3 PM	Postage $
Time In ☐ AM ☐ PM	Military ☐ 2nd Day ☐ 3rd Day	Return Receipt Fee
Weight lbs. ozs.	Int'l Alpha Country Code	COD Fee / Insurance Fee
No Delivery ☐ Weekend ☐ Holiday	Acceptance Clerk Initials	Total Postage & Fees $

DELIVERY (POSTAL USE ONLY)

Delivery Attempt Mo. Day	Time ☐ AM ☐ PM	Employee Signature
Delivery Attempt Mo. Day	Time ☐ AM ☐ PM	Employee Signature
Delivery Date Mo. Day	Time ☐ AM ☐ PM	Employee Signature

☐ WAIVER OF SIGNATURE *(Domestic Only)* Additional merchandise insurance is void if waiver of signature is requested. I wish delivery to be made without obtaining signature of addressee or addressee's agent (if delivery employee judges that article can be left in secure location) and I authorize that delivery employee's signature constitutes valid proof of delivery.

NO DELIVERY ☐ Weekend ☐ Holiday

Customer Signature

CUSTOMER USE ONLY

METHOD OF PAYMENT:

Express Mail Corporate Acct. No. X920016

Federal Agency Acct. No. or Postal Service Acct. No.

FROM: (PLEASE PRINT) PHONE 858 509 4000

JOHN R. WETHERELL
PILLSBURY WINTHROP LLP
11682 EL CAMINO REAL STE 200
SAN DIEGO CA 92130-2092

TO: (PLEASE PRINT) PHONE ( )

MAIL STOP - PETITION
ASSISTANT COMMISSIONER FOR
PATENTS
PO BOX 1450
ALEXANDRIA VA 22313-1450

L 014357-0278749

PRESS HARD.
You are making 3 copies.

FOR PICKUP OR TRACKING CALL 1-800-222-1811 www.usps.com

EMS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of:

FANG FANG

Serial No.: 09/555,446

Filed: AUGUST 16, 2000

Title: MULTIVALENT RECOMBINANT ANTIBODIES FOR TREATING HRV INFECTIONS

Group Art Unit: To be assigned

Examiner: To be assigned

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. §1.10

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as "Express Mail" in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Mail Stop Petition Box 1450, Alexandria, VA 22313-1450 on August 18, 2003.

Suzanne Lloy Simpson

TRANSMITTAL LETTER

Honorable Commissioner of
Patents and Trademarks
Mail Stop Petition
Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith for filing are the following:

1. Petition to Revive Unintentionally Abandoned Application;
2. Reply/Amendment/Letter;
3. Response to Office Communication;
4. Response to Communication regarding Revocation of Power of Attorney and New Power of Attorney and Change of Address; and
5. Return Postcard.

Please charge our deposit account in the amount of **$1,635.00**, which includes the extensions of time fee and Petition to Review filing fee. If the fee submitted is incorrect or if any other fees are due in connection with this submission, please charge any such fee or credit any overpayment to Deposit Account No. 50-2212, Order No. **014357-0278749**.

Respectfully submitted,

PILLSBURY WINTHROP LLP

By: _____

John R. Wetherell
Registration No. 31,678
Tel.: (858) 509-4022
Fax: (858) 509-4010

Attorney Ref. No.: 014357-0278749

PILLSBURY WINTHROP LLP
11682 El Camino Real, Suite 200
San Diego, CA 92130-2593

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of:

FANG FANG

Serial No.: 09/555,446

Filed: AUGUST 16, 2000

Title: MULTIVALENT RECOMBINANT ANTIBODIES FOR TREATING HRV INFECTIONS

Group Art Unit: To be assigned

Examiner: To be assigned

Certificate of Express Mailing

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as "Express Mail" in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Mail Stop Petition Box 1450, Alexandria, VA 22313-1450 on August 18, 2003.

Suzanne Lloy Simpson

PETITION TO REVIVE UNINTENTIONALLY ABANDONED APPLICATION UNDER 37 C.F.R. §1.137(b)

Honorable Commissioner of
Patents and Trademarks
Mail Stop Petition
Box 1450
Alexandria, VA 22313-1450

Sir:

The above-identified application became abandoned for failure to timely file a response within the statutory period of six months from the mailing date of the Office Communication mailed on December 5, 2001.

Applicants hereby petition for revival of this application. Pursuant to 37 C.F.R. § 1.137(b), the following items are filed herewith:

(1) Petition fee under 37 CFR § 1.17(m);

(2) Reply/Amendment/Letter Transmittal;

(3) Response to Office Action;

(4) Petition and Response fee; and

(5) Return postcard.

Applicants note that no terminal disclaimer is required because this application was filed after June 8, 1995.

Applicants hereby state that the entire delay in filing the required reply from the due date for the reply until the filing of a grantable petition under 37 C.F.R. § 1.137(b) was unintentional.

In view of the above, Applicants submit that all of the requirements of 37 C.F.R. § 1.137(b) have been met. Therefore, Applicants respectfully request that this petition be granted. Favorable action on the merits is also earnestly solicited.

Please charge our deposit account in the amount of **$1,635.00**, which includes the extensions of time fee and Petition to Review filing fee. If the fee submitted is incorrect or if any other fees are due in connection with this submission, please charge any such fee or credit any overpayment to Deposit Account No. 50-2212, Order No. **014357-0278749**.

If questions arise relating to this petition, the Examiner is hereby invited to contact the undersigned to discuss the same.

Respectfully submitted,

PILLSBURY WINTHROP LLP

By: _____

John R. Wetherell
Registration No. 31,678
Tel.: (858) 509-4022
Fax: (858) 509-4010

Attorney Ref. No.: 014357-0278749

PILLSBURY WINTHROP LLP
11682 El Camino Real, Suite 200
San Diego, CA 92130-2593

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of:

FANG FANG

Serial No.: 09/555,446

Filed: AUGUST 16, 2000

Title: MULTIVALENT RECOMBINANT ANTIBODIES FOR TREATING HRV INFECTIONS

Group Art Unit: To be assigned

Examiner: To be assigned

Certificate of Express Mailing

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as "Express Mail" in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Mail Stop Petition Box 1450, Alexandria, VA 22313-1450 on August 18, 2003.

Suzanne Lloy Simpson

SEQUENCE LISTING TRANSMITTAL

Transmitted herewith in the above-identified application are the following documents:

1. Copy of Notice to Comply;
2. Amendment/Response to Notice to Comply;
3. Statement Under 37 CFR § 1.821(f) and (g);
4. Sequence Listing in computer readable format; and
5. Sequence Listing in paper format.

Respectfully submitted,

PILLSBURY WINTHROP LLP

By: _____

John R. Wetherell
Registration No. 31,678
Tel.: (858) 509-4022
Fax: (858) 509-4010

Attorney Ref. No.: 014357-0278749

PILLSBURY WINTHROP LLP
11682 El Camino Real, Suite 200
San Diego, CA 92130-2593

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of:

FANG FANG

Serial No.: 09/555,446

Filed: AUGUST 16, 2000

Title: MULTIVALENT RECOMBINANT ANTIBODIES FOR TREATING HRV INFECTIONS

Group Art Unit: To be assigned

Examiner: To be assigned

Certificate of Express Mailing

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as "Express Mail" in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Mail Stop Petition Box 1450, Alexandria, VA 22313-1450 on August 18, 2003

Suzanne Cloy Simpson

STATEMENT UNDER 37 C.F.R. §1.821(f) and (g)

The contents of the paper copy of the SEQUENCE LISTING and computer readable form submitted herewith are the same. The paper copy of the SEQUENCE LISTING and the computer readable form of the SEQUENCE LISTING do not add new matter.

Respectfully submitted,

PILLSBURY WINTHROP LLP

By: _____

John R. Wetherell
Registration No. 31,678
Tel.: (858) 509-4022
Fax: (858) 509-4010

Attorney Ref. No.: 014357-0278749

PILLSBURY WINTHROP LLP
11682 El Camino Real, Suite 200
San Diego, CA 92130-2593

SEQUENCE LISTING

<110> PERLAN THERAPEUTICS
FANG, FANG

<120> MULTIVALENT RECOMBINANT ANTIBODIES FOR TREATING HRV INFECTIONS

<130> 014357-0278749

<140> 09/555,446
<141> 2000-08-16

<150> PCT/US98/25422
<151> 1998-11-30

<150> US 60/067,119
<151> 1997-12-01

<150> US 60/083,046
<151> 1998-04-24

<150> US 60/090,632
<151> 1998-06-25

<160> 15

<170> PatentIn Ver. 3.1

<210> 1
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human peptide residues

<400> 1
Gln Thr Ser Val Ser
1 5

<210> 2
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human peptide residues

<400> 2
Ser Cys Asp Gln Pro Lys
1 5

<210> 3
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human peptide residues

<400> 3
Lys Glu Leu Leu Leu Pro Gly Asn Asn Arg
1 5 10

<210> 4
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human peptide residues

<400> 4
Pro Asp Gly Gln Ser Thr Ala Lys
1 5

<210> 5
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: linker sequence

<400> 5
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15

<210> 6
<211> 24
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: hinge region sequence

<400> 6
Pro Gln Pro Gln Pro Lys Pro Gln Pro Gln Pro Gln Pro Gln Pro Lys
1 5 10 15

Pro Gln Pro Lys Pro Glu Pro Glu
20

<210> 7
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human complementary peptide sequence

<400> 7
Val Cys Arg His Arg
1 5

<210> 8
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human complementary peptide sequence

<400> 8
Gly His Arg Cys Leu
1 5

<210> 9
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human complementary peptide sequence

<400> 9
Leu Gly Leu Val Thr Gly
1 5

<210> 10
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human complementary peptide sequence

<400> 10
Arg Thr Leu Val Gly Phe
1 5

<210> 11
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human complementary peptide sequence

<400> 11
Pro Val Val Pro Arg Gln Glu Gln Leu Leu
1 5 10

<210> 12
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human complementary peptide sequence

<400> 12
Phe Leu Asn Glu Asp Gly Pro Leu Leu Ala
1 5 10

<210> 13
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human complementary peptide sequence

<400> 13
Phe Ser Cys Ser Leu Pro Ile Arg
1 5

<210> 14
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: human complementary peptide sequence

<400> 14
Gly Ile Pro Val Ser Cys Arg Phe
1 5

<210> 15

<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer sequence

<400> 15
ccctcatagt taagcgtaac g 21

Docket No.: 014357-0278749

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of:
FANG FANG

Serial No.: 09/555,446

Filed: AUGUST 16, 2000

Title: MULTIVALENT RECOMBINANT ANTIBODIES FOR TREATING HRV INFECTIONS

Group Art Unit: Not yet assigned

Examiner: Not yet assigned

Certificate of Express Mailing

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as "Express Mail" in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Mail Stop Petition Box 1450, Alexandria, VA 22313-1450 on August 18,2003

Suzanne Lloy Simpson

RESPONSE TO COMMUNICATION AND AMENDMENT

United States Patent and Trademark Office
Attn: Box Sequence
P.O. Box 1450
Alexandria, VA 22312-1450

Sir:

Please amend the subject application as follows:

IN THE SPECIFICATION

On page 4, line 22, please replace with:

---Target No. 1: Residues 1-5 QTSVS (**SEQ. ID. NO. 1**)---

On page 4, line 23, please replace with:

---Target No. 2: Residues 24-29 SCDQPK (**SEQ. ID. NO. 2**)---

On page 4, line 24, please replace with:

--- Target No. 3: Residues 40-49 KELLLPGNNR (**SEQ. ID. NO. 3**)---

On page 4, line 25, please replace with:

--- Target No. 4: Residues 70-77 PDGQSTAK (**SEQ. ID. NO. 4**)---

On page 12, lines 29-32, please replace with:

---The VH and VL genes are liked by an artificial linker $(GGGGS)_3$ (**SEQ.ID.NO. 5**) to form scFv fragment [VH-$(GGGGS)_3$.VL] as in Batra, et al., 1990, and the scFv fragment is subcloned into a pBlueScript vector (Stratagene, CA).---

On page 13, lines 11-13, please replace with:

---scFv fragments are linked with the pentamerization domain of COMP via a hinge region (**SEQ.ID.NO. 6**) to give rise to the following fragment: scFv-hinge $[(PQ)_2$ $PK(PQ)_4$ $PKPQPK(PE)_2]$- Pentamerization domain (COMP aa 28-72).---

On page 18, lines 1-3, please replace with:

---Sequence carried by the selected phage are then determined using the Sequence kit (United States Biochemical) with the primer 5'-CCCTCATAGTTAAGCGTAACG-3'(**SEQ. ID. NO. 15**) (Koivunen, E., et al. (1993) J. Bio. Chem 268:20205-20210).---

On page 18, line 18 , please replace with:

---Target No. 1: VCRHR (**SEQ. ID. NO. 7**) & GHRCL (**SEQ. ID. NO. 8**)---

On page 18, line 19 , please replace with:

---Target No. 2: LGLVTG (**SEQ. ID. NO. 9**) & RTLVGF (**SEQ. ID. NO. 10**)---

On page 18, line 20 , please replace with:

---Target No. 3: PVVPRQEQLL (**SEQ. ID. NO. 11**) & FLNEDGPLLA (**SEQ. ID. NO. 12**)---

On page 18, line 21 , please replace with:

---Target No. 4: FSCSLPIR (**SEQ. ID. NO. 13**) & GIPVSCRF (**SEQ. ID. NO. 14**)---

REMARKS

The application and claims have been amended to refer to updated sequence identifiers in conjunction with Applicant's response to the Notice to Comply mailed December 5, 2001. A marked-up copy of the original page of the application bearing the modified paragraph reflect the amendments in red ink.

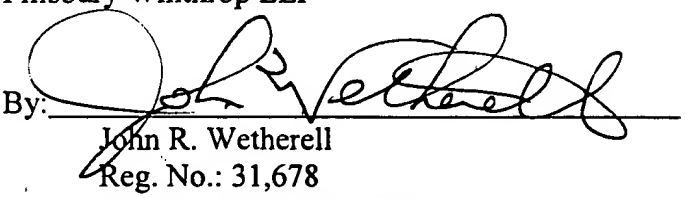
Submitted herewith are substitute paper and computer readable copies of the Sequence Listing. An executed statement under 37 C.F.R 1.821(f) and (g) that the paper and computer readable copies of the Sequence Listing are identical and that the substitute Sequence Listing does add new matter is submitted herewith.

The amendments to the specification to add sequence identifiers and the Sequence Listing were made to correct several informalities. As such no new matter has been added and entry of the amendments and Sequence Listing is respectfully requested.

If the fee authorized is incorrect or if any other fees are due in connection with this submission, please charge any such fee or credit any overpayment to Deposit Account No. 50-2212.

Respectfully submitted,

Pillsbury Winthrop LLP

By: 

John R. Wetherell
Reg. No.: 31,678
Tel. No.: (858) 509-4022
Fax No.: (858) 509-4010

STATEMENT UNDER 37 C.F.R. §1.821(f) and (g)

In connection with the sequence listing submitted concurrently herewith, the undersigned hereby states that:

1. the content of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c), (e), (f) and (g), or § 1.825(d) and (b), respectively, are the same.
2. the submission, filed herewith in accordance with 37 C.F.R. § 1.821(g), does not include new matter.

Respectfully submitted,

Pillsbury Winthrop LLP

By: _____

John R. Wetherell
Reg. No.: 31,678
Tel. No.: (858) 509-4022
Fax No.: (858) 509-4010

PILLSBURY WINTHROP LLP
11682 El Camino Real,
Suite 200
San Diego, CA 92130

containing one or more peptide fragments having affinity for other peptide fragments of the same or substantially the same amino acid sequence. The polymerization domain determines the configuration of the multimeric protein complex (*e.g.*, as dimer, trimer, tetramer, pentamer, or others). Examples of polymerization domains include, but are not limited to, coiled-coil domains such as described and defined in Lupas *et al.*, 1991, Science 252:1162-1164, and alpha-helical sequences as described and defined in Eisenberg, D. *et al.*, (1986) Protein 1:16-22, Ho and DeGrado (1987) J.A.Chem.Soc. 109:6751-6758, Regan and DeGrado, (1988) Science 241:976-978, and Hill *et al.*, (1990) Science 249:543-546. A polymerization domain can be a naturally occurring peptide or can be designed and synthesized artificially. A naturally occurring polymerization domain may be modified to generate other polymerization domains (*see* Harbury *et al.* Science 262:1401-1407, 1993). In even more preferred embodiments, the coiled-coil domain comes from the leucine zipper region of transcription factor GCN4, the tetramerization domain of p53, or the N-terminal residues (aa 20-80) of cartilage oligomeric matrix protein. In another even more preferred embodiment, the alpha-helical sequences contain single helices or helix-turn-helix as summarized in Plückthun and Pack, (1997) Immunotechnology 3:83-105, incorporated by reference herein.

HRV bind to ICAM-1 in four regions. In preferred embodiments, the multivalent peptides bind to three or more (preferably four or more) amino acids in the following regions of human ICAM-1. These are referred to as the target sequences or target peptides hereinafter:

Target No. 1: Residues 1-5: QTSVS (SEQ. ID. NO 1)
Target No. 2: Residues 24-29: SCDQPK (SEQ. ID. NO 2)
Target No. 3: Residues 40-49: KELLLPGNNR (SEQ. ID. NO 3)
Target No. 4: Residues 70-77: PDGQSTAK (SEQ. ID. NO 4)

The multivalent recombinant antibodies and peptides of this invention bind to cellular receptors for rhinovirus with high affinity. In a preferred embodiment, they have an apparent affinity constant for the cellular receptors of no less than 10^8 M^{-1}. In a further preferred embodiment, they have an apparent affinity constant for the cellular receptors of no less than 10^9 M^{-1}. In an even further preferred embodiment, they have an apparent affinity constant for the cellular receptors of no less than 10^{10} M^{-1}.

By "apparent affinity constant" is meant the ratio of [Ab-Ag]/[Ab][Ag] when the antibody-antigen binding reaction reaches equilibrium. [Ab-Ag], [Ab], [Ag] are the

ICAM-1 and LDLR used to raise antibody in this invention can be of any species origin as long as the antibody raised is able to reduce the binding of HRV to their human host cells.

C. Cloning the single chain Fv (scFv) region of the mAb and humanizing the antibody

Single chain Fv fragments against ICAM-1 and LDLR are cloned from hybridomas against each antigen.

One way (a preferred way) of cloning the VH and VL fragments is to amplify them by polymerase chain reaction (PCR) from a hybridoma directed against ICAM-1 or LDLR. Alternatively, the Fab fragment of the desired antibody can be cloned from a combinatorial immunoglobulin library in phage λ by panning using the purified ICAM-1 and LDLR as the antigens (Kang *et al.*, 1991, Methods 2:111-118; Barbas and Lerner, 1991, Methods 2:119-124). The polypeptides of VH and VL can be connected via a synthetic linker to form a single-chain Fv fragment (scFv), or one scFv fragment can dimerize with another to form either diabodies (Holliger *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:6444-6448) or chelating recombinant antibodies (CRAbs) (Neri *et al.*, 1995, J. Mol. Biol. 246:367-373).

The amino acid sequences of the VH and VL domains can be modified based on the original antibody to increase the affinity to antigens or improve the yield of production in E coli or yeast. The origin of the antibody can be of any mammals, including, but are not limited to, human, mouse, rat and rabbit. When the original antibody is isolated from a species other than human, the VH and VL domains of the antibody can be humanized, such as using the methods in U.S. Patent 5,530,101.

Total RNA is isolated from each hybridoma and is converted into cDNA by AMV reverse transcriptase. The VH and VL genes are amplified by PCR using different combinations of degenerate primers in the Ig-Primer kit (Novagen, WI) according to the manufacturer's protocol, or using the primers as described in Larrick & Fry, 1991, Methods 2:106-110. The resulting VH and VL fragments are cloned into a TA cloning vector (Invitrogen, CA) and sequenced. The sequences of multiple clones are compared and the consensus sequences are used to construct the scFv fragments. The VH and VL genes are linked by an artificial linker $(GGGGS)_3$ (SEQ. ID. NO 5) to form scFv fragment [VH-$(GGGGS)_3$-VL] as in Batra *et al.*, 1990, and the scFv fragment is subcloned into a pBlueScript vector (Stratagene, CA). Methods of making svFv fragment are also described in U.S. Patents 5,571,894 and 5,608,039.

The cloned scFv sequence can be humanized to make it less immunogenic or nonimmunogenic to a human host. The humanization of antibody can be carried out as described in U.S. Patent 5,530,101. The sequences of scFv fragments can also be modified to increase the yield of production while still maintaining their antigenic specificity, such as changing the genetic codons or including a signal sequence as described in U.S. Patent 5,648,237.

D. Constructing multivalent recombinant antibody genes

Methods of making multivalent recombinant antibodies are summarized in Plückthun and Pack, (1997) Immunotechnology 3:83-105, incorporated by reference herein.

A pentavalent recombinant antibody is preferred. scFv fragments are linked with the pentamerization domain of COMP via a hinge region (SEQ. ID. NO 6) to give rise to the following fragment: scFv-hinge[$(PQ)_2$ $PK(PQ)_4$ $PKPQPK(PE)_2$]-Pentamerization domain (COMP aa 28-72).

The pentamerization domain of COMP is amplified from plasmid p3b-COMP (Efimov *et al.*, 1994, FEBS letters 341:54-58) by PCR (Tomschy *et al.*, 1996, EMBO J. 14:3507-3514). The amino acid sequence of this COMP domain can be modified to increase the stability of the complex. For example, the Lys-29 and Ala-30 can be changed to cysteine residues (Terskish *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:1663-1668).

There are numerous sequences known to those skilled in the art as suitable for the hinge region. Therefore, the one listed here is only an example. The COMP pentamerization domain can be linked with the hinge and scFv by PCR and ligation using the standard molecular biology techniques. The whole fragment is subcloned into pTrc/His vector (Invitrogen, CA) to generate a bacteria expression plasmid pTrc/scFv-COMP.

E. Expressing multivalent recombinant antibodies in *E. coli*

The scFv-comp protein is expressed in *E. coli* as described in Terskiskh *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:1663-1668. Briefly, *E. coli* transformed with the plasmid pTrc/scFv-COMP is grown in culture until OD600 = 0.5, then 1 mM isopropyl b-D-thiogalactoside is added to induce protein synthesis, followed by a 4 hr incubation at 30^0C. Bacteria are harvested by centrifugation, lysed by three-rounds of freezing/thawing. The recombinant antibody protein is purified from the bacterial lysate by a nickel column (Qiagen).

Sequences carried by the selected phage are then determined using the Sequenase kit (United States Biochemical) with the primer 5'-CCCTCATAGTTAAGCGTAACG-3'(Koivunen, E. *et al.* (1993) J. Bio. Chem. 268:20205-20210).

(SEQ.ID.NO 15)

Example 4: Identifying binding peptides to the target sequences in human ICAM-1 by molecular recognition theory and target complementary library technology (TCLT)

According to the molecular recognition theory, peptides with opposite hydropathic profiles bind to each other. One example is that a pair of peptides encoded by the sense and anti-sense strands of DNA bind to each other specifically (*see* Provisional Application Serial No. 60/083046 filed April 24, 1998, incorporated by reference herein in its entirety; and Blalock, J. E. (1990) TIBTECH 8:140-144), and they are known as complementary peptides.

Complementary peptides to the target sequences in human ICAM-1 are encoded by the antisense strand of human ICAM-1 gene in either 5'-3' or 3'-5' orientation. Therefore, for each target peptide, there are two complementary sequences, and both have specific affinity for the target peptide. The sequences of the complementary peptides for each target sequence in human ICAM-1 are listed in the following:

(SEQ.ID.NO 7) (SEQ.ID.NO 8)
Target No. 1: VCRHR & GHRCL
(SEQ.ID.NO 9) (SEQ.ID.NO 10)
Target No. 2: LGLVTG & RTLVGF
(SEQ.ID.NO 11) (SEQ.ID.NO 12)
Target No. 3: PVVPRQEQLL & FLNEDGPLLA
(SEQ.ID.NO 13) (SEQ.ID.NO 14)
Target No. 4: FSCSLPIR & GIPVSCRF

These complementary peptides (and their homologs) and peptides containing such may have the ability to bind to human ICAM-1 molecule at the target sequences. The affinity of a complementary peptide to its target sequence can be improved by optimizing the peptide sequence via a computer program, or by selecting peptides from a target complementary peptide library as described in Provisional Application Serial No. 60/083046.

Example 5: Construction of multivalent peptides against human ICAM-1

One way of making a multivalent peptide is to link multiple copies of a single peptide or multiple copies of different peptides in tandem repeats to create molecules with the following structures:

[peptide A-(linker)-]$_n$ (n >or= 2) or

[peptide A-(linker)-peptide B-(linker)-]$_n$ (n >or= 2)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1995 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 270, No. 22, Issue of June 2, pp. 13216–13224, 1995
Printed in U.S.A.

Kinetics and Thermodynamics of Virus Binding to Receptor

STUDIES WITH RHINOVIRUS, INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1), AND SURFACE PLASMON RESONANCE*

(Received for publication, February 7, 1995, and in revised form, March 31, 1995)

José M. Casasnovas and Timothy A. Springer‡

From the Harvard Medical School, Department of Pathology, The Center for Blood Research, Boston, Massachusetts 02115

We have studied the kinetics and thermodynamics of a virus interacting with its receptor using human rhinovirus serotype 3 (HRV3), soluble intercellular adhesion molecule-1 (ICAM-1, CD54) containing Ig superfamily domains 1–5 (sICAM-1), and surface plasmon resonance. There were two classes of binding sites for sICAM-1 on HRV3, each comprising about 50% of the total sites, with association rate constants of 2450 ± 300 and 134 ± 11 M^{-1} s^{-1}. These rates are low, consistent with binding to a relatively inaccessible site in the rhinovirus canyon. By contrast, three monoclonal antibodies bound to sICAM-1 with a single rate constant of 17,000–48,000 M^{-1} s^{-1}. The dissociation rate constant for HRV3 was 1.7 ± 0.1 × 10^{-3} s^{-1}, giving calculated dissociation constants of 0.7 ± 0.1 and 12.5 ± 1.2 μM. Agreement was good with saturation binding in solution, which showed two sites of similar abundance with K_D of 0.55 ± 0.2 and 5.7 ± 2.0 μM. A bivalent chimera of ICAM-1 with the IgA1 Fc region bound with K_D = 50 and 410 nM, showing 17-fold enhanced affinity. Lowering pH from 8.0 to 6.0 reduced affinity by approximately 50-fold, primarily by reducing the on rate. Thermodynamic measurements showed that binding of ICAM-1 to HRV3 is endothermic, by contrast to binding to monoclonal antibody. The heat that is absorbed of 3.5 and 6.3 kcal/mol for the two classes of ICAM-1 binding sites may contribute to receptor-mediated disruption of virions, which has an activation energy of about 42 kcal/mol.

Human rhinoviruses are small, non-enveloped RNA viruses of the picornavirus family that have a capsid of icosahedral symmetry and are 300 Å in diameter (1–3). The outer part of the capsid is constructed from 60 copies of viral coat proteins VP1, VP2, and VP3; VP4 is located on the inner face of the proteinaceous capsid. It has been proposed that the receptor binding site is located in a depression or "canyon" encircling the 5-fold icosahedral vertices and that five receptor binding sites are present around each of these vertices (4). Cryoelectron microscopy, site-directed mutagenesis, and saturation binding studies have confirmed these hypotheses (5, 6, 19). The residues implicated in the interaction with the receptor are buried in the canyon, which has been hypothesized to make the receptor binding site inaccessible to antibodies and to protect the viral receptor binding site from immune surveillance (4). It may also be important that antibody binding sites have a bigger footprint than the receptor binding site (8), and thus mutations in regions outside but adjacent to the receptor binding site can contribute to the immunological diversity among serotypes without affecting receptor binding.

Intercellular adhesion molecule-1 (ICAM-1)[1] is the receptor for the major group of rhinoviruses (9–11). ICAM-1 is a membrane protein with five immunoglobulin superfamily (IgSF) extracellular domains, a hydrophobic transmembrane domain, and a short cytoplasmic domain (12, 13). ICAM-1 is the counter-receptor for the leukocyte integrins LFA-1 and Mac-1 and promotes a wide range of cell interactions important in inflammation (14). ICAM-1 is also utilized as a sequestration receptor for *Plasmodium falciparum*-infected erythrocytes (15). The binding site on ICAM-1 for rhinovirus is located primarily in the N-terminal or first IgSF domain, with perhaps some contribution from the second IgSF domain (16, 17). A recombinant soluble form of ICAM-1 truncated at the membrane that contains all five IgSF domains (sICAM-1) has been shown to inhibit rhinovirus infection (18) and induce irreversible modification of the viral capsid *in vitro* (7, 19, 20). High efficiency in rhinovirus neutralization has been obtained with multivalent ICAM-1 Ig chimeras (20).

The equilibria, kinetics, and thermodynamics of virus binding to receptors is interesting from both biological and mechanistic points of view. The affinity constant is a measure of the goodness of fit between a virus and a receptor and is important in determining, together with the number of receptors bound per virion, the effective or apparent affinity that governs the amount of virus binding to cells in equilibrium. Affinity constants have previously been measured for binding of purified influenza virus hemaglutinin to sialic acid (21). The kinetics of virus:receptor interactions are also of great importance, both because few processes reach equilibrium *in vivo*, and because the kinetic constants are of inherent interest and may lead to insights into the virus:host cell interaction and to useful comparisons with other receptor:ligand interactions. However, we are unaware of previous measurements of the kinetic constants for virus:receptor interactions. Binding of picornaviruses to soluble as well as cell surface receptors destabilizes these viruses and promotes disruption of the protein capsid and release of the viral RNA, which is thought to be important in the infection pathway (7, 19, 20, 22–24). Thermodynamic measurements of virus:receptor interaction may yield insights into the mechanism of destabilization.

To address these questions, we have applied surface plasmon resonance, using the BIAcore instrument (25), to the study of virus:receptor interactions (26). This technology has allowed us

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: The Center for Blood Research, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115.

[1] The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; sICAM-1, soluble ICAM-1; IgSF, immunoglobulin superfamily; CHO, Chinese hamster ovary; MSX, L-methionine-sulfoximine; mAb, monoclonal antibody; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; HRV3, human rhinovirus serotype 3.

Exhibit 2

to measure binding of soluble, monomeric ICAM-1 to rhinovirus, and dissociation from rhinovirus, in real time. The equilibrium constant derived from the kinetic constants, and direct measurement of the equilibrium constant by separation of the reactants by sedimentation, reveal two classes of sites that differ in affinity and association rate constant. There are dramatic differences in kinetic constants between binding of soluble ICAM-1 to rhinovirus and to mAb. The effect of lowered pH and receptor dimerization on kinetics and equilibria have been examined. Furthermore, we have determined the kinetic constants at different temperatures and found that heat is absorbed upon receptor binding, which has implications for destabilization of the virion in receptor-mediated disruption.

MATERIALS AND METHODS

sICAM-1—sICAM-1, the extracellular portion of ICAM-1 containing IgSF domains 1–5, was expressed from the mutant cDNA clone Y452E/F* (18) using a recombinant baculovirus in SF9 cells as described previously (22). For expression of sICAM-1 after co-amplification with glutamine synthetase (GS) (27), Y452E/F* was introduced into the unique *Xho*I-*Not*I site of PBJ5-GS (28), to produce pBJ5-GS/IC1–5D. A CHO cell mutant (CHO Lec3.2.8.1.) (29) with highly restricted glycosylation was transfected with PBJ5-GS/IC1–5D using calcium phosphate. After transfection, cells were grown in selective medium with 10% dialyzed serum. Selective medium was essentially as recommended (30) and contained minimum Eagle's medium without glutamine (Life Technologies, Inc.) supplemented with sodium pyruvate (Life Technologies, Inc.), minimum Eagle's medium-non-essential amino acid solution (Life Technologies, Inc.), glucose (0.3% final concentration), glutamic acid and asparagine (60 μg/ml), nucleosides (7 μg/ml), and L-methionine-sulfoximine (MSX). 25 μM MSX was used in the first round of selection. Clones secreting sICAM-1 to the extracellular medium were detected by enzyme-linked immunosorbent assay (31). After the first round of selection, clones were amplified using higher MSX concentrations (100 to 500 μM MSX). Clone CHO-ICAM-1(5D) 37.2.3.2 was seeded in roller bottles with expanded surface (Corning), and cells were grown to confluence using selective media (150 ml) containing 500 μM MSX and 10% dialyzed serum. Serum concentration was reduced to 5% when cells grew to confluence and the roller bottle maintained at 37 °C and 2 rpm for about 1 month. Medium was changed when the protein concentration was about 80 μg/ml as monitored by sandwich enzyme-linked immunosorbent assay.

sICAM-1 was purified from the cell supernatant by immunoaffinity chromatography with R6.5 mAb Sepharose as described (26), with the modification that size exclusion chromatography was with Sephadex G-200 in PBS, pH 8.0 (150 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 4.86 mM Na_2HPO_4). Less nonspecific binding of sICAM-1 in BIAcore was found after purification at pH 8.0 than at pH 7.4. Protein concentration was determined from the extinction coefficient at 280 nm of 0.8 ml/mg cm (20). The molecular weight for the unglycosylated protein (49,600) was used to calculate molarity.

sICAM-1 expressed in baculovirus and mutant CHO cells has similar molecular weight (M_r) (~60,000) and glycosylation content (not shown). sICAM-1 differing in glycosylation had similar affinity for rhinovirus.[2]

IC1–5D/IgA was purified as described previously (20).

Virus Obtention—HRV3 was purified, washed free of bovine serum albumin, and concentration determined spectrophotometrically as described previously (22, 26).

Immobilization of Rhinovirus and Antibodies in BIAcore—HRV3 was covalently immobilized to the dextran surface of certified CM5 sensor chips via primary amino groups, using the amine coupling kit (Pharmacia Biosensor AB (26)). Briefly, carboxylate groups on the dextran were activated by injection of a mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide. Purified virus diluted with 10 mM sodium acetate, pH 5.7, was injected through the activated surface. Ethanolamine hydrochloride, pH 8.5, was injected after virus immobilization to block unreacted *N*-hydroxysuccinimide esters. Running buffer in BIAcore used for washing and the dissociation phase was PBS, pH 8.0, and was identical to the buffer that sICAM-1 was obtained in after Sephadex G-200 chromatography. After each cycle of sICAM-1 association and dissociation, HRV3 was regenerated with 3 pulses of 2 or 3 min of 25 mM MES buffer, pH 6.0, separated by about 1 min of PBS, pH 8.0. Polyclonal rabbit anti-mouse Fc γ antibody (Pharmacia) was immobilized (about 8,000 resonance units) using the amine coupling kit as recommended by the manufacturer.

Analysis of the Binding Data in BIAcore—Sensorgrams recorded during the interaction of sICAM-1 with immobilized virus or captured anti-ICAM-1 antibodies were analyzed by the linear transformation method to obtain the kinetic constants (32), using the equation $dRU/dt = k_a$ [sICAM-1] RU_{max} − RU (k_a [sICAM-1] + k_d), where RU is resonance units. Linear analysis of the binding data uses the slope (k_s) of a plot of dRU/dt *versus* R to determine the association rate constant (k_a). The equation $k_s = k_a$ [sICAM-1] + k_d allows determination of k_a from a plot of k_s *versus* sICAM-1 concentration. Several sICAM-1 concentrations were required, as described in the figure legends.

Nonlinear curve fitting was carried out with the BIA evaluation 2.0 program, using models of one site or two independent sites to calculate association rate constants.

The portion of the sensorgram that corresponds to the dissociation of sICAM-1 from the rhinovirus or antibody surfaces was analyzed to obtain k_d. The slope of a plot of $\ln(R1/R_n)$ *versus* time yielded k_d, where $R1$ is the initial RU and R_n is RU with time. The sensorgrams obtained for the highest ligand concentration were analyzed to minimize rebinding during dissociation. When k_d was determined at different flow rates, no change was detected.

Saturation Binding of sICAM-1 to HRV3 in Solution—sICAM-1 was labeled with [^{35}S]methionine and cysteine, purified, and concentration determined by A_{280} as described (22). A mixture of labeled and unlabeled sICAM-1 (0.2–10 μM final concentration) was incubated with HRV3 (2.1×10^9 virions/μl) in 75 μl for 1 h at 20 or 15 °C, and 70 μl was subjected to sucrose gradient sedimentation for 1 h at 40,000 rpm at 4 °C and fraction collection and scintillation counting as described (22). sICAM-1 that sedimented with the virus in the region of 120–149 S, and input sICAM-1, were used to calculate the concentration of bound and free sICAM-1 in the 75-μl reaction mixture. No sedimentation of sICAM-1 was observed when virus was omitted or when R6.5 mAb to ICAM-1 was added.

RESULTS

Interaction of sICAM-1 with Immobilized Rhinovirus in BIAcore—Human rhinovirus serotype 3 (HRV3) was covalently coupled through amino groups to dextran that is immobilized on the gold film on the surface of the BIAcore sensor chip. Changes in density of the solution within the vicinity of the gold film (<1 wavelength) can be measured, because this affects the refraction of polarized light and the angle of the absorption maximum by the plasmon electrons of the gold film (25). The density in the vicinity of the gold film increases as virus is covalently immobilized, or ICAM-1 is noncovalently bound. Reactions occur within a 0.12-nl dextran layer on a sensor chip in a 60-nl flow chamber and are measured by the change in RU. About 10,000 RU of virus were typically covalently immobilized, corresponding to a concentration of 12.3 μM within the dextran layer and a total of 1.5 fmol. Specificity was examined by injecting ICAM-1 at 20 °C through flow chambers that contained dextran that was either covalently coupled to HRV3 or mock-coupled in the absence of HRV3 (Fig. 1*A*). sICAM-1 (6 μM) bound to immobilized HRV3 (454 RU), but little or no binding was obtained when no virus was present (36 RU). Binding of sICAM-1 (5 μM) to the viral surface was inhibited 85% when the sICAM-1 was preincubated with R6.5 monoclonal antibody (6 μM) for 15 min at 37 °C (not shown). Reproducible binding of sICAM-1 to immobilized HRV3 was obtained, and the virus remained intact as shown by binding of the same amount of sICAM-1 and no change in the base line after regeneration, for at least 12 successive cycles of binding and regeneration (26). Regeneration, *i.e.* dissociation of the bound sICAM-1 between successive cycles of injection of sICAM-1, was achieved at 20 °C with 3 pulses of 25 mM MES buffer, pH 6.0.

Determination of the association kinetic constant (k_a) requires measurement of association kinetics at several different sICAM-1 concentrations, whereas dissociation kinetics can be measured at all concentrations with typically the highest concentration yielding the most reproducible data; sensorgrams

[2] J. M. Casasnovas and T. A. Springer, unpublished results.

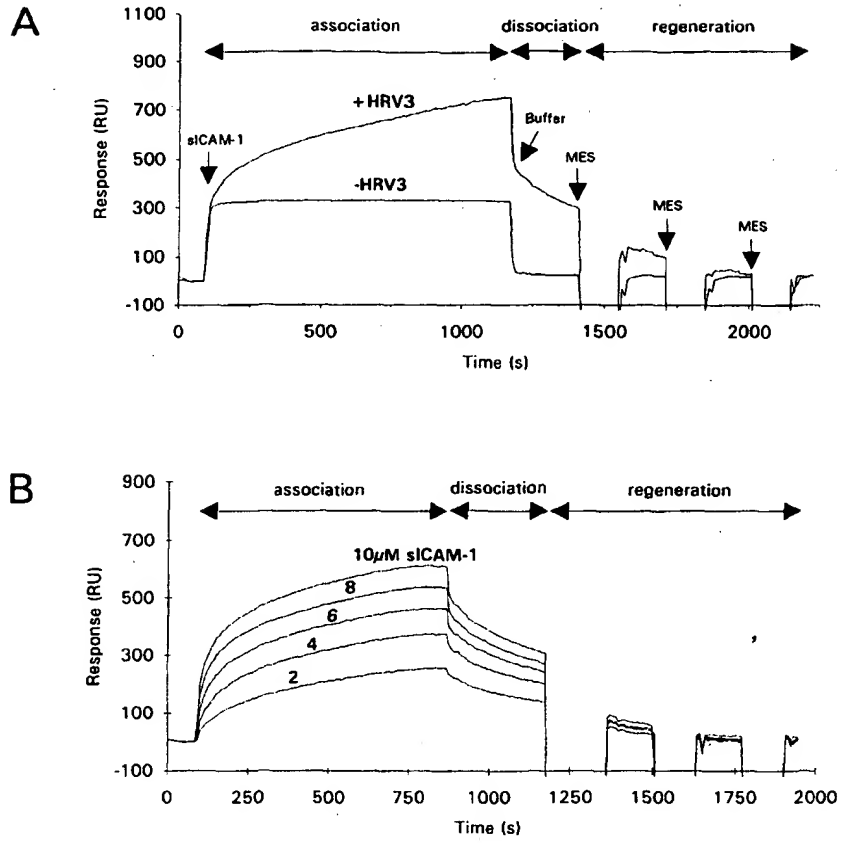


FIG. 1. **Sensorgrams recording interaction of sICAM-1 with rhinovirus in BIAcore.** Association during injection with sICAM-1 and dissociation during injection of buffer lacking sICAM-1 are marked by a gradual increase and decrease in resonance units (*RU*), respectively. Changes in buffer are marked by sharp changes in RU because of changes in buffer density. Regeneration in the final segments of the plots with pH 6.0 MES buffer occurs after 1400 or 1200 s and is marked by a return to the preinjection base line. *A*, overlay plot of sensorgrams, normalized to the same preinjection RU, recorded where sICAM-1 (6 μM) was injected at a flow rate of 2 μl/min and 20 °C through a carboxymethylated dextran surface that had been coupled without (−) or with (+) HRV3 (~10,000 RU). This was followed by an injection of PBS for 3.5 min. during which sICAM-1 dissociated from the surface. The matrix was then regenerated with three pulses (3 min) of 25 mM MES buffer, pH 6.0. *B*, overlay plot of sensorgrams obtained from successive cycles of association, dissociation, and regeneration. sICAM-1 (2, 4, 6, 8, and 10 μM) was successively injected through the sensor chip with immobilized rhinovirus at a flow rate of 3 μl/min at 20 °C. HRV3 was regenerated with 3 pulses (3, 2, and 2 min) of 25 mM MES buffer, pH 6.0.

showing association and dissociation obtained in a typical experiment are overlaid in Fig. 1*B*. The rate of ICAM-1 binding (dRU/d*t*) slowed during the association phase, but binding of sICAM-1 (RU) did not plateau, showing that equilibrium was not reached during the injection period. The HRV3 was stable during the experiment, because a very similar base line was recorded at the beginning and end of each cycle. The ratio of the maximal RU of bound sICAM-1 to the RU of covalently immobilized HRV3 was 0.075. Calculations using the M_r of virions (8.16×10^6) and sICAM-1 (60,000), and 60 binding sites per virion, yield binding of sICAM-1 that is 14–18% of the theoretical maximum and may indicate that this is the percentage of binding sites that are accessible or remain active after coupling to dextran.

Plots of the rate of association (dRU/d*t*) of sICAM-1 with HRV3 *versus* the amount of sICAM-1 that had associated (RU) were biphasic (Fig. 2*A*). Two classes of binding sites were distinguished by linear transformation of the data (Fig. 2*A*) and by nonlinear curve fitting (data not shown). The latter method yielded poor fits for one binding site ($\chi^2 = 45$) and excellent fits for two sites ($\chi^2 = 1.8$). Both the linear and nonlinear methods gave similar k_a values.

Interaction of sICAM-1 with immobilized mAb to ICAM-1 was measured for comparison to results with HRV3. The plot for the association of sICAM-1 with R6.5 mAb (and other mAb, data not shown) showed a single straight line (Fig. 2*B*).

The rates (k_s) for the first phase (Fig. 3*A*) and second phase (Fig. 3*B*) of association of sICAM-1 with HRV3 were plotted against sICAM-1 concentration. The slope of the line yielded k_a with a correlation coefficient $r > 0.99$. The intersection with the *y* axis is one method of determining k_d and yielded similar results for the first and second phases of association of 1.7×10^{-3} s^{-1} and 1.8×10^{-3} s^{-1}, respectively. Plots of k_s *versus* sICAM-1 concentration for association of sICAM-1 with mAb also yielded straight lines with $r = 0.99$.

Plots of dissociation of sICAM-1 from HRV3 *versus* time which have a slope = k_d yielded a straight line (Fig. 3*C*). No change in k_d was observed when the injection rate during dissociation was increased to 8 μl/min (not shown). Plots of dissociation of sICAM-1 from mAb were also linear and yielded k_d that did not change with injection rate. Invariance with injection rate for dissociation from HRV3 and mAb suggested there was no significant rebinding of dissociated sICAM-1.

Kinetic Rate Constants—The kinetic rate constants for sICAM-1 interaction with HRV3 were highly reproducible. Representative data obtained with four different HRV3 preparations and with four different preparations of sICAM-1 from CHO cells and insect cells are shown in Table I. The association rate constant for the first phase of association, $k_{a,1}$, was 2450 ± 300 M^{-1} s^{-1}. This is slow relative to other studied association rate constants, including mAb (see below). The rate for $k_{a,2}$ was 18-fold slower than for $k_{a,1}$. The k_d rate constant was $1.67 \times 10^{-3} \pm 0.1 \times 10^{-3}$ s^{-1}. The equilibrium dissociation constant (K_D) was calculated from k_d/k_a, using $k_{a,1}$ to obtain K_D1 and $k_{a,2}$ to obtain K_D2. These values were 0.69 ± 0.09 μM and 12.5 ± 1.2 μM, respectively.

The amount of binding to the two classes of sites was determined from the amount of bound sICAM-1 at the inflection point in the binding curves at high (near saturation) concentrations of sICAM-1 and also from Scatchard plots of the amount of binding to the two classes of sites. Both methods suggested that the sites with higher and lower k_a comprise about 40–50% and 50–60% of the total sites, respectively.

We obtained comparative kinetic data on three mAb that bind to ICAM-1. The mAb bind to sites on ICAM-1 overlapping or nearby to the site that binds HRV, since all three mAb block binding of HRV to rhinovirus (10), and the epitopes have been mapped to IgSF domains 1 or 2 (16) (Table II). The k_a of

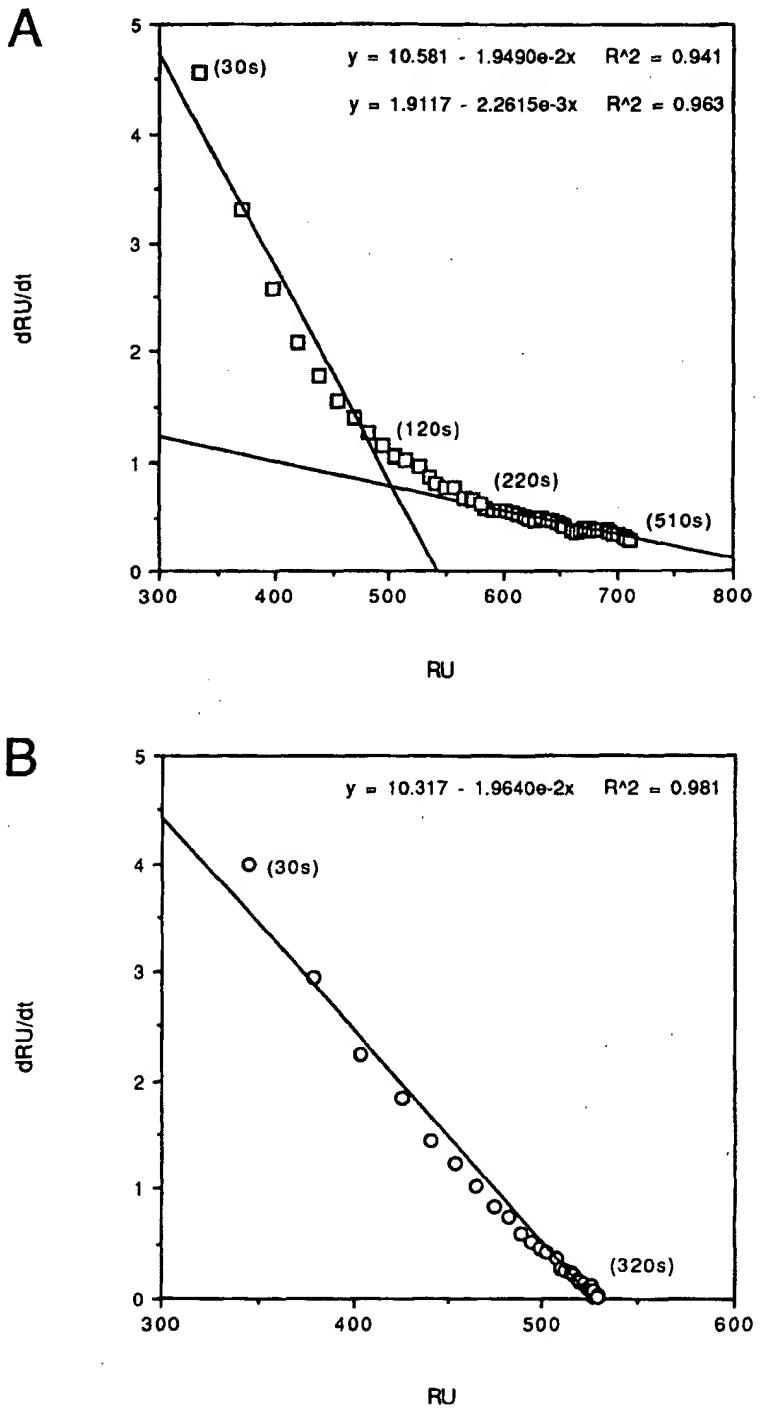


FIG. 2. **Linear transformation of the association of sICAM-1 with immobilized HRV3 and anti-ICAM-1 R6.5 mAb.** The value of dRU/d*t* every 10 s during the association phase was plotted *versus* RU. Selected time intervals after the beginning of the association with sICAM-1 are shown in *parentheses*. *A*, sICAM-1 (8 μM) was injected through a sensor chip with immobilized HRV3 at 3 μl/min and 20 °C as in Fig. 1*B*. *B*, R6.5 mAb to ICAM-1 (25 μg/ml) was injected for 12 min followed by sICAM-1 (1 μM) for 13 min in PBS at 20 °C and 3 μl/min through a sensor chip containing immobilized rabbit anti-mouse Fc antibody.

sICAM-1 for the three mAb was 17,000–47,800 M^{-1} s^{-1} or 7–20-fold faster than $k_{a,1}$ for HRV3. By contrast, the k_d values for HRV3 and the three mAb were quite similar.

Affinity in Solution—We compared our measurements with classical techniques for measuring affinity in solution. HRV3 (1.2 nM) was incubated with varying amounts of ^{35}S-sICAM-1 for 1 h at 20 or 15 °C, chilled on ice, and virus-bound sICAM-1 was separated from free sICAM-1 by sucrose gradient sedimentation at 4 °C. The Scatchard plot (Fig. 4) showed two classes of binding sites that differed in affinity. K_D1 and K_D2 determined from three independent experiments at 20 °C (±S.D.) were 0.55 ± 0.2 μM and 5.7 ± 2.0 μM, respectively. $K_{D,1}$ and $K_{D,2}$ at 15 °C were 0.84 μM and 6.9 μM. The high affinity sites comprised 48% of the total at both temperatures.

Effect of pH on the Interaction of sICAM-1 with Rhinovirus—Rhinoviruses are labile to pH lower than 6.0 (33). Lowering pH below 7.0 decreases the amount of sICAM-1 that associates with rhinovirus as shown by co-sedimentation in sucrose gradients (22). We tested the effect of lowered pH on the kinetics and equilibria of ICAM-1 binding to HRV3 (Table III). The affinity of HRV3 for sICAM-1 (inverse of $K_{D,1}$) was 1.8-fold lower at pH 7 than pH 8. A decrease from pH 7 to pH 6.5 resulted in a further 4.6-fold drop in affinity, primarily due to a 3.2-fold drop in $k_{a,1}$. At pH 6 there was a substantial further

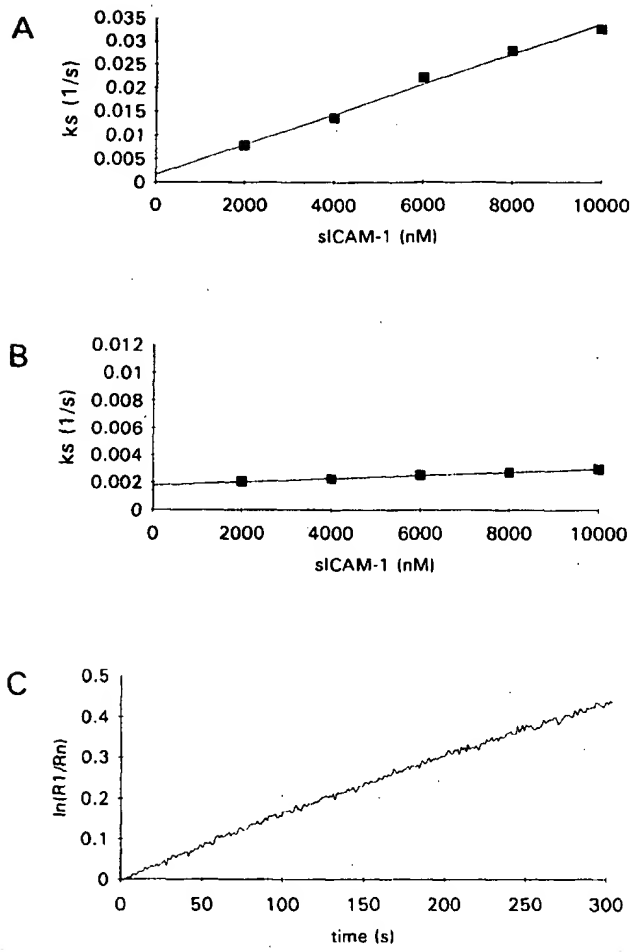


FIG. 3. **Representative plots for determination of the kinetic rate constants for sICAM-1 interaction with HRV3.** *A* and *B*, linear transformations of the association phase of sensorgrams as shown in Fig. 2*A* were used to obtain the slopes (k_s) from the first (*A*) and second (*B*) part of the association phase and k_s was plotted *versus* sICAM-1 concentration. The slope of these curves obtained by linear regression equals k_a. *C*, a representative linear transformation of the sensorgram from the dissociation phase obtained with 10 μM sICAM-1. *R1*, resonance units at beginning of dissociation; *Rn*, resonance units at the indicated time.

drop in affinity, as shown by less binding of sICAM-1 at the end of the association phase with 10 μM sICAM-1 than at higher pH (Table III). The k_d was 2-fold faster at pH 6.0 than at pH 6.5, and too little sICAM-1 was bound for measurement of k_a. The affinity roughly dropped 6-fold from pH 6.5 to 6.0, since the same amount of sICAM-1 was bound at the end of the association phase at pH 6.5 with 5 μM sICAM-1 (52 RU) as at pH 6.0 with 30 μM sICAM-1 (48 RU). Thus, from pH 8 to pH 6 there was a marked drop in affinity of approximately 50-fold. There was no disruption of HRV3 at pH 6 to 8 at 20 °C, since there was no change in the base line of immobilized rhinovirus, and since binding of sICAM-1 was reproducible in repeat experiments with the same sensor chip, and since full sICAM-1 binding activity was obtained in binding experiments at pH 8 that followed those at pH 6 (data not shown). Indeed, it should be noted that the ICAM-1 binding sites on HRV3 were routinely regenerated at the end of every association and dissociation cycle with pulses of pH 6 buffer.

Effect of ICAM-1 Dimerization on the Interaction with Rhinovirus—A dimeric ICAM-1 chimera, with ICAM-1 IgSF domains 1–5 fused to the hinge, CH2, and CH3 domains of the IgAI Fc region, has increased efficacy in neutralizing and disrupting rhinovirus (20); however, its affinity has not been measured previously. We measured the kinetics of the interaction of this IC1–5D/IgA chimera with HRV3 (Table IV). The kinetics were measured in terms of the concentration of the ICAM-1 moiety, *i.e.* for the dimeric chimera 2 × the molar concentration. The $k_{a,1}$ and $k_{a,2}$ kinetic constants were 1.6- and 2.8-fold faster for IC1–5D/IgA than for sICAM-1, respectively, whereas k_d was 7.6-fold slower. This resulted in a 17-fold higher affinity for IC1–5D/IgA than for sICAM-1.

Thermodynamics of the Interaction of sICAM-1 with HRV3 and R6.5 mAb—Thermodynamics of the interactions were determined by measuring kinetic constants at temperatures ranging from 10 to 25 °C (Table V and Fig. 5). For the interaction of sICAM-1 with both HRV3 and R6.5 mAb, k_a and k_d increased with increasing temperature. For HRV3, $k_{a,1}$ and $k_{a,2}$ increased more than k_d, whereas for R6.5 mAb k_a increased less than k_d. The data fit straight lines on Arrhenius plots (Fig. 5) except for the k_d for HRV3 at 25 °C. This value was disregarded in the plot because of decrease in the HRV3 RU base line at the end of each cycle at 25 °C, suggesting virus disruption; at 30 °C substantial disruption of HRV3 occurred in the presence of sICAM-1 (26). The $K_{D,1}$ and $K_{D,2}$ for HRV3 decreased by 1.2- and 1.4-fold as temperature increased from 10 to 20 °C. By contrast, the K_D for R6.5 mAb increased from 10 to 20 °C by 1.3-fold.

The activation energy (E_a) for association and dissociation was determined from Arrhenius plots (Fig. 5). The highest E_a were for $k_{a,2}$ for sICAM-1 binding to HRV3 and k_d for dissociation of sICAM-1 from R6.5 mAb. The E_a for dissociation of sICAM-1 was markedly higher with R6.5 mAb than with HRV3.

The enthalpy ($\Delta H°$) and free energy ($\Delta G°$) for the association of sICAM-1 with HRV3 and R6.5 at 20 °C were determined from the activation energies and affinity constants, respectively (Table VI). The HRV3-sICAM-1 interaction is an endothermic process, as shown by the positive $\Delta H°$ for the reaction. Association of sICAM-1 with the second class of binding sites on HRV3 was markedly more endothermic than with the first class. Association of sICAM-1 with R6.5 mAb was exothermic, with a negative $\Delta H°$. The entropy term ($T\Delta S°$) was positive for both interactions, but was higher for the interaction of sICAM-1 with HRV3 than with R6.5.

DISCUSSION

We have measured the kinetic constants for the interaction of a virus with its receptor. We adapted surface plasmon resonance to the study of virus-receptor interactions, and found it to be an accurate method for measuring kinetic constants, with standard deviations that were almost always less than 10% of the mean and with results that were highly reproducible with independent viral and receptor preparations. The utility of the method was enhanced by development of conditions that allowed at least 12 successive cycles of sICAM-1 binding and regeneration to be carried out with a single rhinovirus-sensor chip preparation (26). No disruption of the virus was detected in the determination of the kinetic and dissociation constants at 20 °C and below, as monitored by changes in the base line or amount of sICAM-1 bound.

The association rate constants for binding of sICAM-1 to HRV3 were low, 2450 and 130 M^{-1} s^{-1}. Since we are unaware of any previous measurements of the kinetic constants for virus binding to receptors, it is useful to compare kinetic measurements for other protein-protein interactions. Association rate constants for antibody binding to proteins in solution or on the cell surface range from 10^4 to 2 × 10^6 M^{-1} s^{-1} (34), consistent with our range of 2–5 × 10^4 M^{-1} s^{-1} for three ICAM-1 mAbs.

TABLE I

Kinetic and dissociation constants for sICAM-1 and HRV3

Four representative experiments are shown that used four different preparations of sICAM-1 (from insect cells in experiments 1 and 2 and from Lec 3.2.8.1 CHO cells in experiments 3 and 4) and four different preparations of HRV3. A range of concentrations of sICAM-1 from 1 to 10 μM was injected in each experiment as in Fig. 1*B*. $k_{a,1}$ and $k_{a,2}$ were obtained from the analysis of the first and second part of the biphasic association phase, respectively. Dissociation constants are the average of measurements from the dissociation phase of the two highest sICAM-1 concentrations. K_D were calculated from the kinetic constants; $K_{D,1} = k_d/k_{a,1}$ and $K_{D,2} = K_d/k_{a,2}$. Standard deviations are in parentheses.

	$k_{a,1}$	$k_{a,2}$	$k_d \times 10^3$	$K_{D,1}$	$K_{D,2}$
	$M^{-1}\ s^{-1}$		s^{-1}	μM	
Exp. 1	2,910	121	1.75	0.60	14.4
Exp. 2	2,420	131	1.50	0.62	11.4
Exp. 3	2,400	151	1.74	0.72	11.5
Exp. 4	2,063	132	1.70	0.82	12.8
Average	2,450 (300)	134 (11)	1.67 (0.1)	0.69 (0.09)	12.5 (1.2)

TABLE II

Kinetic and dissociation constants for sICAM-1 and mAb

A polyclonal rabbit anti-mouse Fc antibody (Pharmacia) was coupled to dextran in the sensor chip. Anti-ICAM-1 monoclonals (32 μl, 20 μg/ml) and sICAM-1 (36 μl; 50, 100, 150, 200, 250, or 300 nM) were consecutively injected through the surface containing the rabbit antibody for 8 and 9 min, respectively, at 4 μl/min at 25 °C in PBS, pH 8.0. The rabbit anti-mouse Fc was regenerated by injection of 8 μl of 100 mM HCl after each cycle. k_a is from a single or two experiments, with range in parenthesis. k_d is the average and s.d. of measurements from the dissociation phase from three cycles where 300, 500, and 750 nM sICAM-1 were injected at 4, 8, and 30 μl/min, respectively.

Antibody	Epitope	k_a	$k_d \times 10^3$	K_D
		$M^{-1}\ s^{-1}$	s^{-1}	nM
R6.5	Domain 2	23,700	1.83 (0.24)	77
LB2	Domain 1	17,100 (1,200)	1.79 (0.09)	105
RR1/1	Domain 1	47,800	1.80 (0.07)	38

On rates for the IgE receptor and insulin receptor are 6×10^4 and 3×10^6 $M^{-1}\ s^{-1}$ (34), respectively, whereas the adhesion molecules CD2 and CD48 interact with an on rate $> 10^5$ $M^{-1}\ s^{-1}$ (35). The latter interaction has an affinity similar to that of ICAM-1 for HRV3. There are two interesting possible explanations for why association of sICAM-1 with rhinovirus proceeds 1–2 orders of magnitude more slowly than typically found for protein:protein interactions. The location of the rhinovirus binding site in a depression in the viral capsid may make it relatively inaccessible and require precise orientation of the ICAM-1 for binding. Alternatively, there may be a requirement for a conformational change in the binding site on rhinovirus for ICAM-1 to bind.

The k_d of 1.67×10^{-3} s^{-1} found for sICAM-1 and HRV3 translates to a $t_{1/2}$ or lifetime of the receptor-ligand bond of 6.9 min. The k_d was not unusual and was similar to that found for three different mAb to ICAM-1.

We found two different kinetic association constants for the sICAM-1 interaction with rhinovirus. This contrasted with the results for association of three mAb with sICAM-1, where a single k_a was found. Of the binding sites on HRV3, 40–50% bound with the faster on rate and the remainder with the slower on rate. Using our kinetic constants, we calculated the equilibrium dissociation constant of sICAM-1 for HRV3. The K_D is 0.69 ± 0.09 μM and 12.5 ± 1.2 μM for the two classes of binding sites. Equilibrium binding measurements in solution yielded a biphasic Scatchard plot. The K_D of 0.55 ± 0.2 μM and 5.7 ± 2.0 μM were in good agreement with the BIAcore results. There were almost equal numbers of low and high affinity sites, as also found with BIAcore.

There are several possible explanations for the two classes of low and high affinity binding sites. One, there could be inherent differences between the structure of binding sites on the rhinovirus capsid. These two classes of binding sites appear to be on the same virion rather than on different classes of virions, because the rhinovirus sedimentation coefficient shifted gradually from 149 to 120 S as increasing amounts of sICAM-1 were added, and two different populations of virions that were altered in sedimentation by different concentrations of sICAM-1 were not seen (22). Binding of "pocket factor" to pockets underlying half of the sites could produce the observed heterogeneity (3). Two, binding of sICAM-1 could induce conformational changes in the rhinovirus capsid that alters the structure of neighboring binding sites. Three, binding of the first 2 or three molecules of sICAM-1 per pentamer might proceed without hindrance, but binding of further sICAM-1 molecules might be impeded. Electron microscopy of a two-domain fragment of ICAM-1 bound to rhinovirus reveals no contacts between neighboring ICAM-1 molecules (5); however, bound ICAM-1 projects outward from the virus and thus might kinetically hinder binding, by requiring more precise orientation for an ICAM-1 molecule to gain access to a neighboring binding site.

Our K_D measurements may be compared with previous determinations of the IC_{50} for inhibition by sICAM-1 of virus binding to cells, infection at high multiplicity of infection or plaque-forming units (7, 18, 20). For HRV3, the IC_{50} for inhibition of binding to purified ICAM-1 on a substrate was 3.5 μM, and the IC_{50} for infectivity at high MOI and in plaque-forming assays was 1.2 μM and 0.4–0.3 μM, respectively (7, 20). The high affinity K_D of 0.7 μM is close to these IC_{50} values and suggests that occupancy of these sites may be sufficient for inhibition of these processes. Inhibition of binding to cells is obtained at lower IC_{50} values of 45 nM (20), suggesting that occupancy of only a few sites is sufficient to inhibit virus attachment. IC_{50} values for HRV54 (18) and HRV14 (20) are lower than for HRV3, and these viruses may have correspondingly lower K_D for sICAM-1.

Multivalency results in an effective increase in affinity. Multivalent chimeric molecules containing IgSF domains 1–2 or 1–5 of ICAM-1 fused to Fc portions of IgA1 or IgM exhibited lower IC_{50} than sICAM-1 in assays of binding to cells, disruption, and plaque-forming units (20). We measured the kinetics and K_D for the IC1–5D/IgA chimera in units of concentration of the ICAM-1 moiety, for fair comparison with sICAM-1. The chimera, like sICAM-1, exhibited two k_a. The $k_{a,1}$ and $k_{a,2}$ were 1.6- and 2.8-fold faster than for sICAM-1, respectively. Theoretically, k_a for sICAM-1 and IC1–5D/IgA are predicted to be the same (34). Bivalent binding is predicted to result in a decreased k_d, in agreement with the finding that k_d was 7.5-fold lower for IC1–5D/IgA than for sICAM-1. The K_D for IC1–5D/IgA were 50 and 411 nM, 17-fold lower than for sICAM-1. This compares with IC_{50} values for IC1–5D/IgA of 7.5-, 12.6-, or 187-fold lower than for sICAM-1 for virus binding, disruption, or plaque-forming units, respectively (20). The IC_{50} values for these three assays were 1.6–38 nM. Compared with the high affinity K_D of 50 nM or average K_D of 230 nM, this suggests that 50% inhibition occurs when substantially less than 50% of the

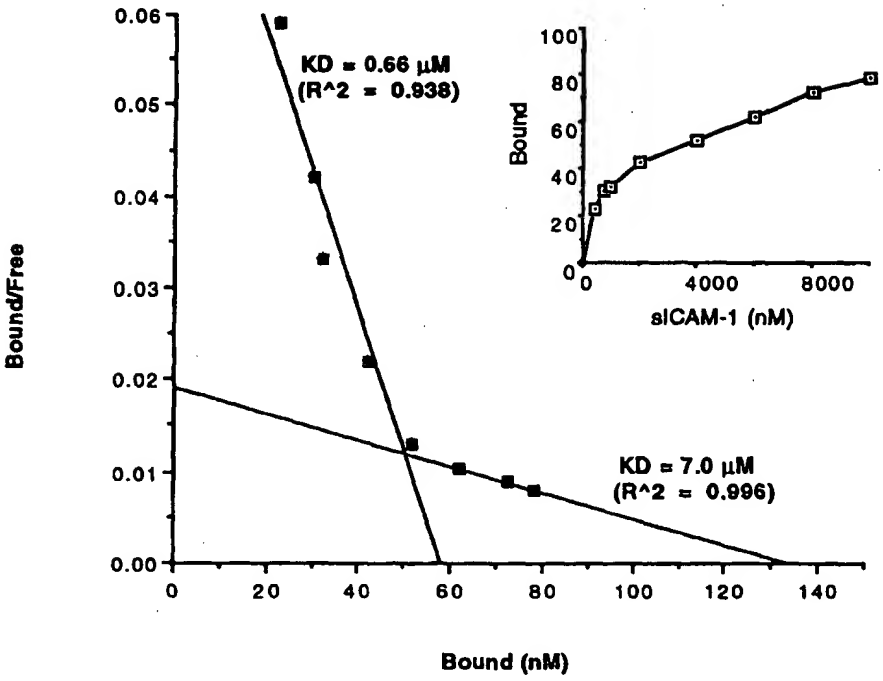


FIG. 4. **Equilibrium binding of sICAM-1 to HRV3 in solution.** HRV3 (2.1×10^9 virions/μl) was incubated with varying concentrations of ^{35}S-sICAM-1 for 60 min at 20 °C, chilled on ice, subjected to sucrose gradient sedimentation, and fractions were scintillation counted. Radioactivity that co-sedimented with the virus was used to calculate bound sICAM-1.

TABLE III

Effect of pH on kinetic and dissociation constants for sICAM-1 and HRV3

Kinetic association constants (k_a) are averages of two experiments with range in parentheses; k_d constants are average of four measurements with S.D. in parentheses. The buffer for injection of ICAM-1 and the dissociation phase was PBS adjusted to the indicated pH. sICAM-1 was at a range of concentrations from 1 to 10 μM, pH 8.0; 1 to 15 μM, pH 7.0; 5 to 30 μM, pH 6.5; and 10 to 30 μM, pH 6.0. $k_{a,1}$, $k_{a,2}$, and k_d were determined and K_D calculated as described in Table I. The last column shows the amount of sICAM-1 that was bound in RU after 13 min of injection at 10 μM. The amount of HRV3 immobilized was 11,000 RU.

pH	$k_{a,1}$	$k_{a,2}$	$k_d \times 10^3$	$K_{D,1}$	$K_{D,2}$	Bound sICAM-1 at 10 μM
	$M^{-1}\,s^{-1}$		s^{-1}	μM		RU
8.0	2,320 (260)	156.3 (24)	1.56 (0.14)	0.67	10.0	353
7.0	1,880 (83)	127.6 (3.9)	2.32 (0.06)	1.23	18.2	214
6.5	580 (200)	ND[a]	3.26 (0.26)	5.60	ND	66
6.0	ND	ND	6.00 (0.06)	ND	ND	32

[a] ND, not determined.

TABLE IV

Kinetic and dissociation constants for sICAM-1 and IC1–5D/IgA

Association rate constants were determined from two different experiments for each molecule using different sensor chips, as described in the legend to Fig. 1*B*. Measurement units are the concentration of ICAM-1 sites, *i.e.* 2 × molar concentration for IC1–5D/IgA. Concentrations injected ranged from 1 to 8 μM for sICAM-1 and 0.5 to 4 μM for IC1–5D/IgA. The experiments were done in parallel at 4 μl/min using PBS, pH 8.0. Dissociation constants were determined from the two highest protein concentrations in each experiment, and one additional experiment with the highest protein concentration at 10 μl/min. The standard deviation is in parentheses.

Sample	$k_{a,1}$	$k_{a,2}$	$k_d \times 10^3$	$K_{D,1}$	$K_{D,2}$
	$M^{-1}\,s^{-1}$			nM	
sICAM-1	2,690 (150)	189 (5.5)	1.67 (0.3)	621	8820
IC1–5D/IgA	4,410 (330)	538 (20)	0.221 (0.02)	50.1	411

sites/virion are occupied. The K_D for binding of HRV14 and HRV15 to HeLa cells is in the range of 10^{-11} M (6). Comparisons with our values of about 10^{-6} M and 10^{-7} M for monovalent and bivalent receptor binding clearly suggest that binding of HRV to cells is highly multivalent.

Both acidic pH and binding to receptor can cause disruption of rhinovirus and may be important in the pathway of infection. However, the combination of lowered pH and receptor binding are not synergistic and not even additive in disruption assays, and less sICAM-1 binding to HRV3 is seen at pH 6 than at pH 7 (19, 22). The affinity of sICAM-1 for HRV3 decreased 8-fold when pH was dropped from 8 to 6.5, primarily as a result of a drop in k_a. This is a very dramatic change in affinity over a small change around neutral pH and may suggest either a charged residue such as histidine that is important in binding or a cooperative change in capsid conformation that affects the receptor binding site.

The thermodynamics of ICAM-1 interaction with rhinovirus may have important implications for virus disruption. Most protein:protein interactions, *e.g.* antibody binding to protein antigens, are exothermic (negative ΔH) and thus have higher affinity as the temperature is decreased (34). The interaction of R6.5 mAb with sICAM-1 was an example of this. By contrast, interaction of HRV3 with ICAM-1 was endothermic; *i.e.* heat was absorbed by the sICAM-1-HRV3 complex. Both interaction with R6.5 mAb and HRV3 resulted in an increase in entropy, consistent with a hydrophobic interaction that results in increased disorder of water molecules. We have previously meas-

TABLE V

Kinetic and association constants for HRV3-sICAM-1 interaction at four different temperatures

sICAM-1 (1–10 or 1–12 μM) was injected at each temperature as shown in Fig. 1*B* and kinetic constants determined as described in Table I. Range (k_a) or standard deviation (k_d) are shown in parentheses.

Temperature	$k_{a,1}$	$k_{a,2}$	$k_d \times 10^3$	$K_{D,1}$	$K_{D,2}$
°C	$M^{-1} s^{-1}$		s^{-1}	μM	
10	1,620 (12)	80.5 (15.9)	1.31 (0.19)	0.81	16.27
15	1,960 (80)	110.5 (3.2)	1.42 (0.14)	0.72	12.85
20	2,410 (8)	141.2 (10.2)	1.61 (0.13)	0.67	11.40
25	3,000 (100)	195.4 (5.8)	2.22 (0.14)	0.74	11.36

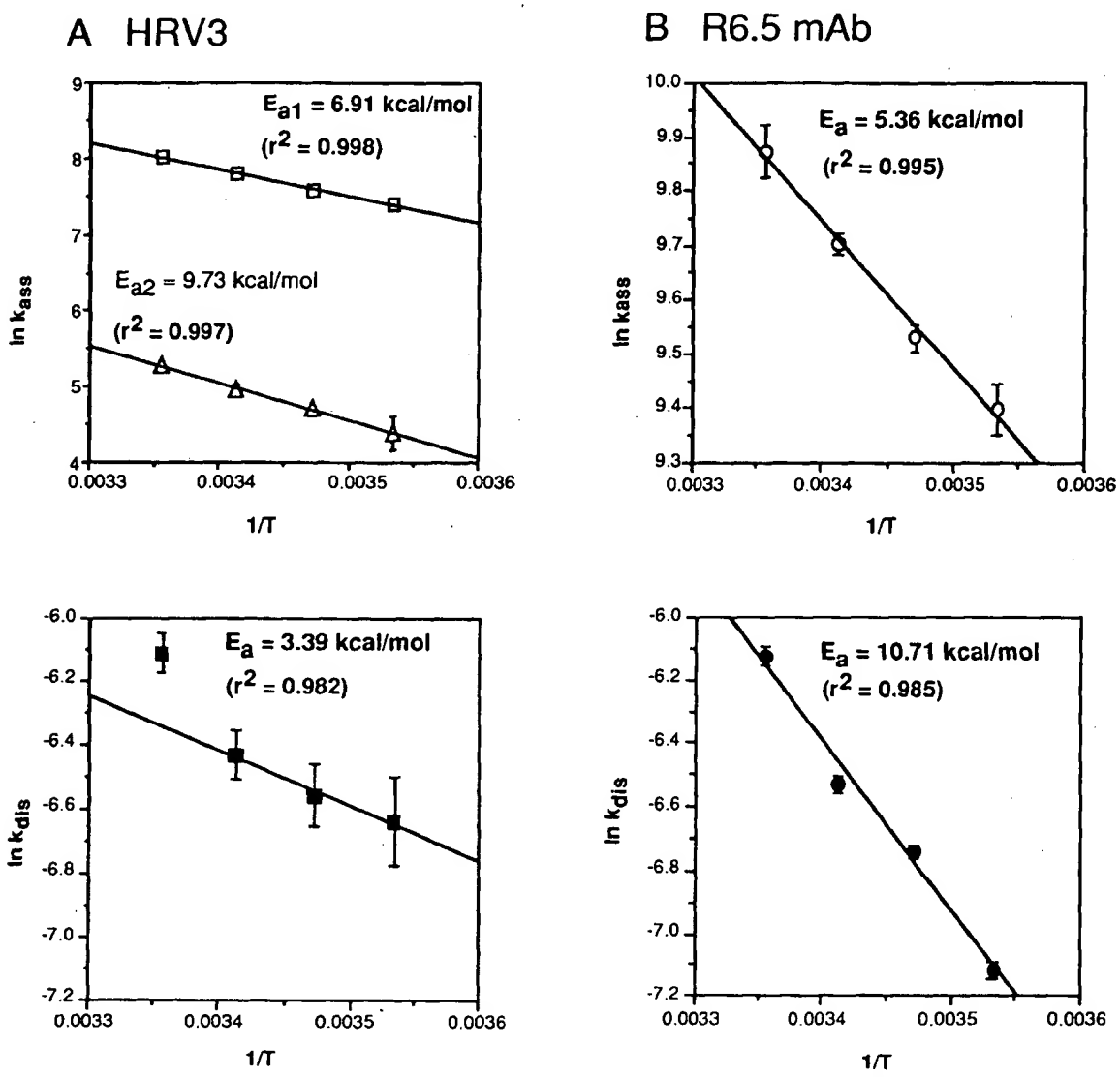


FIG. 5. **Activation energy for k_a and k_d.** Kinetic constants were determined in the BIAcore machine equilibrated to 10, 15, 20, or 25 °C. *A*, for HRV3 association, kinetic constants were determined using ICAM-1 concentrations of 1–12 μM and dissociation constants were determined using the two highest concentrations. Plots obtained with the kinetic constants determined for the first (*open squares*) and second phase (*open triangles*) of the association of sICAM-1 with HRV3 are shown. The mean and range are shown for two independent experiments. *B*, for R6.5 mAb, constants were determined as described in Table II in three different experiments where sICAM-1 was injected at 1, 2, or 4 μl/min through sensor chips with about 900 RU of R.6.5 mAb captured with anti-mouse Fc. Nine concentrations of sICAM-1 between 100 and 500 nM were used in each experiment. k_d was determined from the sensorgrams obtained when sICAM-1 was injected at 350, 400, 450, and 500 nM at flow rate of 4 μl/min. Mean and S.D. are shown for three experiments (k_a) and four dissociations (k_d). Activation energy is obtained from the slope of the plots and T is in Kelvin.

ured the activation energy for disruption of rhinovirus as ~42 kcal/mol (22). Binding of sICAM-1 to rhinovirus accelerates the rate of disruption, and thus partially destabilizes the virion. Part of the heat absorbed when sICAM-1 is bound may contribute to lowering the activation barrier for disruption. The enthalpy for binding to the sites with slower k_a of 6.3 kcal/mol is substantially higher than for binding to sites with faster k_a of 3.5 kcal/mol. Binding to the former class of sites would be predicted to make a greater contribution to disruption because of the greater enthalpy. Since there are a total of 60 sites/

TABLE VI
Thermodynamic parameters

$\Delta H°$ was obtained from the difference between the activation energy for the association and dissociation reaction, which is equivalent to the activation energy for the equilibrium association constant. $\Delta G°$ was determined from the affinity constants at 20 °C ($\Delta G° = -RT \ln K_{eq}$), and $T\Delta S°$ was calculated from the difference between $\Delta H°$ and $\Delta G°$. Data from the first (1) and second (2) phase of the association of sICAM-1 with HRV3 are shown.

Interaction	$\Delta H°$	$\Delta G°$	$T\Delta S°$	$\Delta S°$
	kcal/mol			*cal/°K mol*
ICAM-1 and HRV3 (1)	3.5	−8.3	11.8	40
ICAM-1 and HRV3 (2)	6.3	−6.6	13.0	44
ICAM-1 and R6.5 mAb	−5.4	−9.4	4.0	14

virion, occupation of only a fraction of them would result in an increase in enthalpy similar to that of the activation energy of 42 kcal/mol. It may be significant that binding to the sites with lower enthalpy would precede kinetically binding to the sites with higher enthalpy; this may be important in the pathway of virus disruption and infection. Studies of virus disruption in BIAcore may lead to further insights into the physicochemistry of this process.

REFERENCES

1. Rossmann, M. G., Arnold, E., Erickson, J. W., Frankenberger, E. A., Griffith, J. P., Hecht, H. J., Johnson, J. E., Kamer, G., Luo, M., Mosser, A. G., Rueckert, R. R., Sherry, B., and Vriend, G. (1985) *Nature* **317,** 145–153
2. Kim, S., Smith, T. J., Chapman, M. S., Rossmann, M. G., Pevear, D. C., Dutko, F. J., Felock, P. J., Diana, G. D., and McKinlay, M. A. (1989) *J. Mol. Biol.* **210,** 91–111
3. Oliveira, M. A., Zhao, R., Lee, W.-M., Kremer, M. J., Minor, I., Rueckert, R. R., Diana, G. D., Pevear, D. C., Dutko, F. J., McKinlay, M. A., and Rossmann, M. G. (1993) *Structure* **1,** 51–68
4. Rossmann, M. G. (1989) *J. Biol. Chem.* **264,** 14587–14590
5. Olson, N. H., Kolatkar, P. R., Oliveira, M. A, Cheng, R. H., Greve, J. M., McClelland, A., Baker, T. S., and Rossmann, M. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 507–511
6. Colonno, R. J., Condra, J. H., Mizutani, S., Callahan, P. L., Davies, M., and Murcko, M. A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 5449–5453
7. Greve, J. M., Forte, C. P., Marlor, C. W., Meyer, A. M., Hoover-Litty, H., Wunderlich, D., and McClelland, A. (1991) *J. Virol.* **65,** 6015–6023
8. Smith, T. J., Olson, N. H., Holland Cheng, R., Liu, H., Chase, E. S., Ming Lee, W., Leippe, D. M., Mosser, A. G., Rueckert, R. R., and Baker, T. S. (1993) *J. Virol.* **67,** 1148–1158
9. Greve, J. M., Davis, G., Meyer, A. M., Forte, C. P., Yost, S. C., Marlor, C. W., Kamarck, M. E., and McClelland, A. (1989) *Cell* **56,** 839–847
10. Staunton, D. E., Merluzzi, V. J., Rothlein, R., Barton, R., Marlin, S. D., and Springer, T. A. (1989) *Cell* **56,** 849–853
11. Tomassini, J. E., Graham, D., DeWitt, C. M., Lineberger, D. W., Rodkey, J. A., and Colonno, R. J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 4907–4911
12. Simmons, D., Makgoba, M. W., and Seed, B. (1988) *Nature* **331,** 624–627
13. Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L., and Springer, T. A. (1988) *Cell* **52,** 925–933
14. Springer, T. A. (1995) *Annu. Rev. Physiol.* **57,** 827–872
15. Berendt, A. R., Simmons, D. L., Tansey, J., Newbold, C. I., and Marsh, K. (1989) *Nature* **341,** 57–59
16. Staunton, D. E., Dustin, M. L., Erickson, H. P., and Springer, T. A. (1990) *Cell* **61,** 243–254
17. McClelland, A., DeBear, J., Yost, S. C., Meyer, A. M., Marlor, C. W., and Greve, J. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7993–7997
18. Marlin, S. D., Staunton, D. E., Springer, T. A., Stratowa, C., Sommergruber, W., and Merluzzi, V. (1990) *Nature* **344,** 70–72
19. Hoover-Litty, H., and Greve, J. M. (1993) *J. Virol.* **67,** 390–397
20. Martin, S., Casasnovas, J. M., Staunton, D. E., and Springer, T. A. (1993) *J. Virol.* **67,** 3561–3568
21. Sauter, N. K., Bednarski, M. D., Wurzburg, B. A., Hanson, J. E., Whitesides, G. M., Skehel, J. J., and Wiley, D. C. (1989) *Biochemistry* **28,** 8388–8396
22. Casasnovas, J. M., and Springer, T. A. (1994) *J. Virol.* **68,** 5882–5889
23. Kaplan, G., Freistadt, M. S., and Racaniello, V. R. (1990) *J. Virol.* **64,** 4697–4702
24. Yafal, A. G., Kaplan, G., Racaniello, V. R., and Hogle, J. M. (1993) *Virology* **197,** 501–505
25. Malmqvist, M. (1993) *Nature* **361,** 186–187
26. Casasnovas, J. M., Reed, R. R., and Springer, T. A. (1994) *Methods: A Companion to Methods in Enzymology* **6,** 157–167
27. Davis, S. J., Ward, H. A., Puklavec, M. J., Willis, A. C., Williams, A. F., and Barclay, A. N. (1990) *J. Biol. Chem.* **265,** 10410–10418
28. Gastinel, L. N., Simister, N. E., and Bjorkman, P. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 638–642
29. Stanley, P. (1989) *Mol. Cell Biol.* **9,** 377–383
30. Bebbington, C. R., and Hentschel, C. C. G. (1987) in *DNA Cloning: A Practical Approach* (Glover, D. M., ed) Vol. III, pp. 163–188, IRL Press, Oxford
31. Rothlein, R., Mainolfi, E. A., Czajkowski, M., and Marlin, S. D. (1991) *J. Immunol.* **147,** 3788–3793
32. Karlsson, R., Michaelsson, A., and Mattson, L. (1991) *J. Immunol. Methods* **145,** 229–240
33. Rueckert, R. R. (1990) in *Virology* (Fields, B. N., Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J. L., Monath, T. P., and Roizman, B., eds) pp. 507–547, Raven Press, Ltd., New York
34. Mason, D. W., and Williams, A. F. (1986) in *Handbook of Experimental Immunology: Immunochemistry* (Weir, D. M., ed) Vol. 1, pp. 38.1–38.17, Blackwell Scientific Publications, Oxford
35. van der Merwe, P. A., Brown, M. H., Davis, S. J., and Barclay, A. N. (1993) *EMBO J.* **12,** 4945–4954

JOURNAL OF VIROLOGY, July 1998, p. 6244–6246
0022-538X/98/$04.00+0

Vol. 72, No. 7

The Domain Structure of ICAM-1 and the Kinetics of Binding to Rhinovirus

JOSÉ M. CASASNOVAS, JOANNA K. BICKFORD, AND TIMOTHY A. SPRINGER*

The Center for Blood Research and Harvard Medical School
Department of Pathology, Boston, Massachusetts

Received 10 November 1997/Accepted 9 April 1998

Fragments of intercellular adhesion molecule 1 (ICAM-1) containing only the two most N terminal of its five immunoglobulin SF domains bind to rhinovirus 3 with the same affinity and kinetics as a fragment with the entire extracellular domain. The fully active two-domain fragments contain 5 or 14 more residues than a previously described fragment that is only partially active. Comparison of X-ray crystal structures show differences at the bottom of domain 2. Four different glycoforms of ICAM-1 bind with identical kinetics.

Intercellular adhesion molecule 1 (ICAM-1, CD54) is a cytokine-inducible cell surface receptor that binds to leukocyte integrins LFA-1 and Mac-1 (21). ICAM-1 is also the receptor for the major group of human rhinoviruses (9, 25, 26) and *Plasmodium falciparum*-infected erythrocytes (2, 18). ICAM-1 contains five immunoglobulin superfamily (IgSF) domains and is heavily glycosylated. The binding site for rhinovirus is at the tip of domain 1 in the flexible BC and FG loops and extends about halfway down domain 1 (1, 7, 17, 20, 23). The binding site for LFA-1 is on the edge of domain 1, centered on the C and D strands (1, 7, 8, 24). Soluble ICAM-1 (sICAM) inhibits infection by rhinovirus, both by acting as a competitive inhibitor and by irreversible disruption of the capsid with release of the viral RNA (5, 10, 14, 15). However, ICAM-1 truncated after what was predicted (24) to be the last residue of domain 2, F185, showed markedly reduced inhibition by both mechanisms compared to ICAM-1 containing all five IgSF domains (10, 15). ICAM-1 is unusually heavily glycosylated, and glycosylation is important for solubility (16). There are conflicting reports on the importance of the presence or absence of glycosylation for binding to rhinovirus (13, 16, 17); the influence of different classes of N-glycans, i.e., glycoforms, has not been explored. The kinetics, equilibria, and thermodynamics of ICI-5D binding to rhinovirus have been measured by using surface plasmon resonance (4, 6). The on-rate constant (k_{ass}) for binding of ICI-5D to rhinovirus is much slower than for binding to an antibody. This is consistent with either binding to a relatively inaccessible site in the rhinovirus canyon (19) or a requirement for a conformational change in the virus to permit binding. The k_{ass} is biphasic, and two putative classes of binding sites have K_ds of about 0.7 and 10 μM (6). Here, we determined the kinetics with truncation and glycosylation variants of ICAM-1.

ICAM-1 truncated after residue 185 (IC1-2D/185), 190 (IC1-2D/190), 199 (IC1-2D/199), 268 (IC1-3D), or 452 (IC1-5D) was expressed in insect SF9 cells (IC1-5D/SF9, IC1-3D, and IC1-2D/185), wild-type CHO-K1 cells (IC1-5D/wt), or lectin-resistant CHO Lec 3.2.8.1 cells (IC1-5D/Lec, IC1-2D/199, and IC1-2D/190) (4, 6, 7, 14). Proteins were purified with monoclonal antibody (MAb) R6.5-Sepharose and size exclusion chromatography (6). ICAM-1 containing only one carbohydrate residue per N-linked site (IC1-5D/D) was from the flowthrough from a concanavalin A-Sepharose column that was loaded with IC1-5D/CHOLec treated with 4 mU of endoglycosidase H (endo H)/μg for 4 h at 37°C in 0.1 M phosphate buffer (pH 5.5). Purified human rhinovirus 3 (HRV3) (4) or rabbit anti-mouse Fc IgG (Pharmacia) was linked via amino groups to the dextran surface of BIAcore sensor chips to a density of about 10,000 or 8,000 resonance units, respectively (4). Sensorgrams were subjected to linear transformation to obtain kinetic constants (11). Binding of [^{3}H]leucine-labeled HRV3 to HeLa cells and assay of HRV3 disruption by sucrose gradient centrifugation were done as previously described (15).

IC1-5D/wt was resistant to endo H and sensitive to neuraminidase, whereas IC1-5D/CHOLec and IC1-5D/SF9 were fully susceptible or only partially susceptible to endo H, respectively (Fig. 1). Thus, IC1-5D/wt and IC1-5D/Lec have complex-type and high-mannose N-linked glycans, respectively. Partial resistance of IC1-5D/SF9 to endo H may reflect fucosylation (3), which is absent in CHO Lec 3.2.8.1 cell N glycans (22). IC1-5D/D has only one *N*-acetylglycosamine per N-linked site.

The four different IC1-5D glycoforms exhibited no significant differences in the kinetics of binding to HRV3 (Table 1). Association was biphasic, possibly representing two different classes of binding sites (6). A single dissociation rate constant (k_{diss}) was seen (6).

Compared to IC1-5D and IC1-3D, three different batches of IC1-2D/185 associated with HRV3 with the same kinetics but reproducibly dissociated five- to sixfold faster (Table 2). This agrees with lower activity in inhibition of binding and in inactivation of rhinovirus (10, 15). By contrast, IC1-2D/199 reproducibly reacted with HRV3 with kinetics indistinguishable from those of IC1-5D and IC1-3D (Table 2). At a later stage in this work, we also tested the IC1-2D/190 fragment. Although less data are available, it clearly was at least as active as IC1-5D, IC1-3D, and IC1-2D/199 (Table 2).

The association kinetic constant (k_{ass}) for binding of ICAM-1 fragments to MAbs was 10- to 100-fold faster than that for binding to HRV3 (Table 3). The k_{ass} increased as the number of Ig-like domains in sICAM-1 decreased; this is likely to be related to faster diffusion of the shorter molecules, particularly in the dextran matrix to which the MAbs and virus are linked in BIAcore. The lack of such an effect on k_{ass} for HRV3 is consistent with the finding that this k_{ass} is much slower and, thus, not diffusion limited. Three MAbs to domain 1 of ICAM-1 (12), LB2 (Table 3), 7F7, and MAY.029 (not shown), and

* Corresponding author. Mailing address: The Center for Blood Research and Harvard Medical School Department of Pathology, 200 Longwood Avenue, Boston, MA 02115. Phone: (617) 278-3200. Fax: (617) 278-3232. E-mail: springer@sprsgi.med.harvard.edu.

Exhibit 3

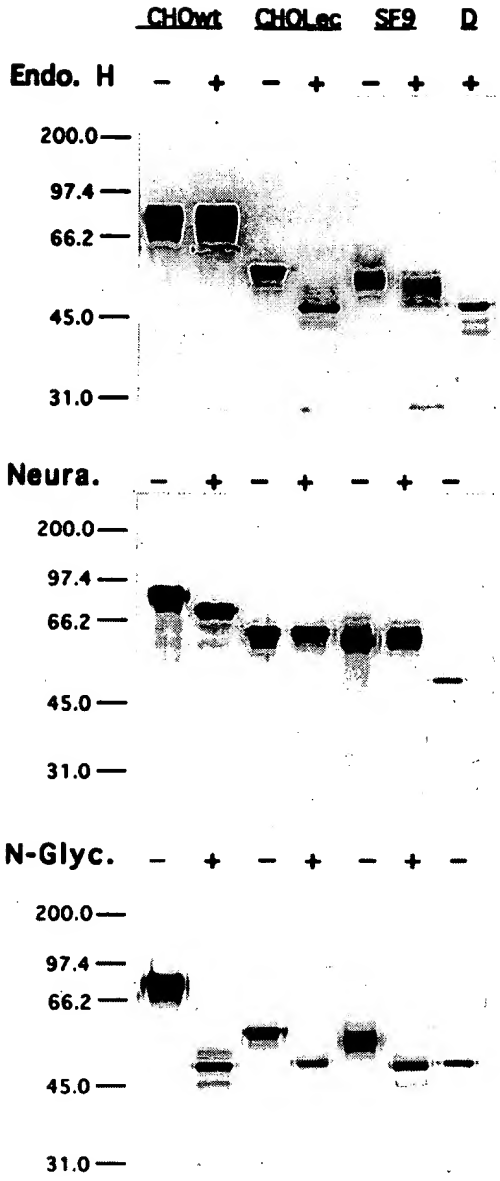


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sICAM-1 glycosylation variants treated with endoglycosidases. IC1-5D obtained from wild-type CHO-K1 (CHOwt), lectin-resistant CHO (CHOLec), or SF9 insect cells were treated in the presence or absence of endo H, neuraminidase (Neura.), or *N*-glycanase (N-Glyc.), whereas the preparation of endo H-deglycosylated material, IC1-5D/D (D), was run without further treatment. Positions of molecular weight markers are shown on the left (molecular weights are in thousands).

one MAb to domain 2, R6.5 (Table 3), dissociated from ICAM-1 fragments with kinetics that were unaffected by the truncation position. By contrast, with MAb RR1/1 to domain 1 of ICAM-1, the IC1-2D/185 fragment dissociated 11.0-fold ± 0.8-fold more rapidly from IC1-2D/185 than from IC1-2D/190, IC1-2D/199, IC1-3D, or IC1-5D. These results confirm the differences between IC1-2D/185 on the one hand and IC1-2D/190 and IC1-2D/199 on the other.

The crystal structures have recently been determined for two independent molecules of IC1-2D/190 (7) and one molecule of

TABLE 1. Kinetic and dissociation constants for binding of sICAM-1 glycosylation variants to HRV3[a]

Protein	$k_{ass}{}^1$ (M^{-1} s^{-1})	$k_{ass}{}^2$ (M^{-1} s^{-1})	k_{diss} (10^3) (s^{-1})	K_{d1} (μM)	K_{d2} (μM)
IC1-5D/wt	1,910 ± 322	115.0 ± 1.3	1.84 ± 0.08	0.96 ± 0.17	16.0 ± 0.7
IC1-5D/SF9	2,670 ± 201	130.0 ± 6.2	1.67 ± 0.16	0.62 ± 0.08	12.9 ± 1.4
IC1-5D/Lec	2,410 ± 268	166.0 ± 22.3	1.70 ± 0.19	0.70 ± 0.11	10.2 ± 1.8
IC1-5D/D	2,500 ± 203	221.0 ± 65.7	1.87 ± 0.30	0.75 ± 0.13	8.4 ± 2.8

[a] Dissociation constants (K_d) were obtained from the kinetic constants as follows: $K_{d1} = k_{diss}/k_{ass}{}^1$ and $K_{d2} = k_{diss}/k_{ass}{}^2$. sICAM-1 concentrations ranged between 1 and 10 μM. All experiments were performed at 20°C with a flow rate of 2 to 3 μl/min. The correlation coefficients obtained in the determination of the kinetic constants by the linear transformation method were about 0.99. The averages and ranges of two or standard deviations of three experiments are shown. The standard deviations of K_{d1} and K_{d2} were calculated as the square root of the sums of the variances of k_{ass} and k_{diss}.

mutIC1-2D/185 that is identical to IC1-2D/185 except that it contains three Asn→Gln mutations that eliminate N-linked glycosylation sites (1). The three structures are quite similar overall, but the position of truncation has a marked effect on the structure of the bottom of domain 2. The backbone hydrogen bonds between F185 in the G strand and residues 98 and 100 in the A′ strand are lost in the 185-residue fragment, and there are significant shifts in the Cα positions of residues 183 and 184 in the G strand, 95 to 97 in the A′ strand and in the connector between the A and A′ strands on the edge of domain 2, 150 to 153 in the EF loop, and 101 and 102 in the A′B loop. Most significantly, Val186 appears to form an important component of the bottom of domain 2, with its hydrophobic side chain packing onto the side chain of Val100 and the hydrophobic portion of the Arg150 side chain. In the structure of the 185-residue fragment, Val186 is missing, the side chain of Phe185 is rotated 180°C, and the bottom of domain 2 is less compact overall. The A′G beta-sheet ladders at the bottom of domains 1 and 2 are structurally homologous in IC1-2D/190, and the Tyr83 and Trp84 side chains occupy orientations similar to those of Phe185 and Val186. Thus, the structure of Phe185 and Val186 in IC1-2D/190 is appropriate for a domain 2-3 connection structurally homologous to the domain 1-2 connection.

Our data show that structural changes at the bottom and side of domain 2 can alter the affinity of rhinovirus interaction with the top portion of domain 1 and with MAb RR1/1 to domain 1. Although this may seem surprising, the conformation of domain 1 has previously been shown to be influenced by

TABLE 2. Kinetic and dissociation constants for binding of sICAM-1 length variants to HRV3[a]

Protein	$k_{ass}{}^1$ (M^{-1} s^{-1})	$k_{ass}{}^2$ (M^{-1} s^{-1})	k_{diss} (10^3) (s^{-1})	K_{d1} (μM)	K_{d2} (μM)
IC1-5D	2,240 ± 439	162 ± 25	1.65 ± 0.06	0.74 ± 0.15	10.2 ± 1.6
IC1-3D	2,060 ± 332	157 ± 49	1.80 ± 0.16	0.87 ± 0.16	11.5 ± 3.7
IC1-2D/199	1,880 ± 361	182 ± 40	1.91 ± 0.12	1.02 ± 0.21	10.5 ± 2.4
IC1-2D/190	2,500	319	1.11	0.44	3.5
IC1-2D/185	2,060 ± 430	130 ± 21	9.27 ± 1.26	4.50 ± 1.12	71.6 ± 15.1

[a] The concentrations of ICAM-1 injected in BIAcore ranged from 1 to 10 μM for IC1-5D, 3D, 2D/199, and 2D/190 and from 4 to 50 μM for 2D/185. Three different preparations of IC1-2D/V185 were used; all gave similar results, which are included in the averages. IC1-3D and IC1-2D/185 were from SF9 cells, and IC1-2D/199 and 2D/190 were from CHO Lec cells; thus, all had similar glycosylation. Values for IC1-5D are from tests run in parallel with those on other variants and include data from both CHO Lec and SF9 cell preparations. The averages and standard deviations shown are from at least three experiments. For other details, see Table 1, footnote *a*.

TABLE 3. HRV-inhibitory MAb binding to length variants of ICAM-1[a]

MAb and sICAM-1	k_{ass} (M^{-1} s^{-1})	k_{diss} (10^3) (s^{-1})	K_d (nM)
R6.5 (D2)			
5D/SF9	23,700	1.83 ± 0.24	77.1
5D/Lec	34,900	1.82 ± 0.14	52.0
2D/185	84,800	1.74 ± 0.15	20.5
2D/199	123,000	2.02 ± 0.20	16.0
2D/190	72,000	1.42 ± 0.01	19.7
LB2 (D1)			
5D/SF9	17,100 ± 1,230	1.79 ± 0.09	105.0
5D/Lec	19,900 ± 710	2.06 ± 0.08	103.0
3D	30,100 ± 2,620	1.82 ± 0.18	61.0
2D/185	40,400 ± 5,090	1.95 ± 0.26	48.0
2D/199	47,500 ± 1,300	2.33 ± 0.12	49.0
RR1/1 (D1)			
5D	47,800	1.80 ± 0.07	38.0
5D/Lec	60,100	1.90 ± 0.01	32.0
3D	85,200	1.74 ± 0.18	20.0
2D/185	197,000	**20.40** ± 2.97	**103.0**
2D/199	196,000	1.78 ± 0.08	9.0
2D/190	214,000	2.10 ± 0.02	9.8

[a] The k_{ass} and k_{diss} determined with BIAcore are for sICAM-1 fragments with captured anti-ICAM-1 MAbs. The k_{diss} was determined from three independent injections of 300, 500, and 750 nM sICAM-1 at 4, 8, and 30 μl/min, respectively. The average and standard deviation for these three injections are shown. No increase in k_{diss} with flow rate was obtained in these experiments. The average and range (k_{ass}) or standard deviation (k_{diss}) of two experiments are shown for the LB2 MAb.

mutagenesis of domain 2, including a conservative Ala178→Gly mutation in the bulge of strand G near the bottom of domain 2 (23). In the three structures captured in crystals, there appear to be no differences in domain 1 that are significant for rhinovirus binding; however, the structure in solution may differ from that in crystals. We suggest that in solution, IC1-2D/185 exists in an equilibrium between two conformations, i.e., (i) an ordered conformation that can bind rhinovirus and corresponds to the conformation that crystallizes and (ii) a conformation in which a portion of domains 1 and 2 is disordered and in which the molecule cannot bind or binds markedly less well to rhinovirus and MAb RR1/1. The IC1-2D/190 fragment has a different conformation at the bottom of domain 2 and appears to be present in an ordered conformation in solution a much higher proportion of the time than the 185-residue fragment. Equilibration between the ordered and disordered forms of the IC1-2D/185 fragment that is fast compared to the rates of binding to and dissociation from HRV3 and antibodies would be consistent with our kinetic data. Our findings emphasize the intricacy and delicacy of protein structure and have important implications for viral receptors and the design of viral antagonists. Furthermore, our results show that domains 1 and 2 of ICAM-1 are sufficient for high-affinity binding to HRV3.

This work was supported by NIH grant AI31921.

REFERENCES

1. **Bella, J., P. R. Kolatkar, C. Marlor, J. M. Greve, and M. G. Rossmann.** 1998. The structure of the two amino-terminal domains of human ICAM-1 suggests how it functions as a rhinovirus receptor and as an LFA-1 integrin ligand. Proc. Natl. Acad. Sci. USA **95**:4140–4145.
2. **Berendt, A. R., A. McDowall, A. G. Craig, P. A. Bates, M. J. E. Sternberg, K. Marsh, C. I. Newbold, and K. Hogg.** 1992. The binding site on ICAM-1 for Plasmodium falciparum-infected erythrocytes overlaps, but is distinct from, the LFA-1-binding site. Cell **68**:71–81.
3. **Butters, T. D., I. Jones, V. A. Clarke, and G. S. Jacob.** 1998. Structural characterization of the N-linked oligosaccharides derived from HIVgp120 expressed in lepidopteran cells. Glycoconj. J. **15**:83–88.
4. **Casanovas, J. M., R. R. Reed, and T. A. Springer.** 1994. Kinetics of receptor and virus interaction and receptor-induced virus disruption: methods for study with surface plasmon resonance. Methods (Orlando) **6**:157–167.
5. **Casasnovas, J. M., and T. A. Springer.** 1994. The pathway of rhinovirus disruption by soluble intercellular adhesion molecule 1 (ICAM-1): an intermediate in which ICAM-1 is bound and RNA is released. J. Virol. **68**:5882–5889.
6. **Casasnovas, J. M., and T. A. Springer.** 1995. Kinetics and thermodynamics of virus binding to receptor: studies with rhinovirus, intercellular adhesion molecule-1 (ICAM-1), and surface plasmon resonance. J. Biol. Chem. **270**: 13216–13224.
7. **Casasnovas, J. M., T. Stehle, J.-H. Liu, J.-H. Wang, and T. A. Springer.** 1998. A dimeric crystal structure for the N-terminal two domains of ICAM-1. Proc. Natl. Acad. Sci. USA **95**:4134–4139.
8. **Fisher, K. L., J. Lu, L. Riddle, K. J. Kim, L. G. Presta, and S. C. Bodary.** 1997. Identification of the binding site in intercellular adhesion molecule 1 for its receptor, leukocyte function-associated antigen 1. Mol. Biol. Cell **8**:501–515.
9. **Greve, J. M., G. Davis, A. M. Meyer, C. P. Forte, S. C. Yost, C. W. Marlor, M. E. Kamarck, and A. McClelland.** 1989. The major human rhinovirus receptor is ICAM-1. Cell **56**:839–847.
10. **Greve, J. M., C. P. Forte, C. W. Marlor, A. M. Meyer, H. Hoover-Litty, D. Wunderlich, and A. McClelland.** 1991. Mechanisms of receptor-mediated rhinovirus neutralization defined by two soluble forms of ICAM-1. J. Virol. **65**:6015–6023.
11. **Karlsson, R., A. Michaelsson, and L. Mattson.** 1991. Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. J. Immunol. Methods **145**:229–240.
12. **Klickstein, L. B., and T. A. Springer.** 1995. CD54 (ICAM-1) cluster report, p. 1548–1550. *In* S. F. Schlossman, L. Boumsell, W. Gilks, J. Harlan, T. Kishimoto, T. Morimoto, J. Ritz, S. Shaw, R. Silverstein, T. Springer, T. Tedder, and R. Todd (ed.), Leucocyte typing. V. White cell differentiation antigens. Oxford University Press, New York, N.Y.
13. **Lineberger, D. W., D. J. Graham, J. E. Tomassini, and R. J. Colonno.** 1990. Antibodies that block rhinovirus attachment map to domain 1 of the major group receptor. J. Virol. **64**:2582–2587.
14. **Marlin, S. D., D. E. Staunton, T. A. Springer, C. Stratowa, W. Sommergruber, and V. Merluzzi.** 1990. A soluble form of intercellular adhesion molecule-1 inhibits rhinovirus infection. Nature **344**:70–72.
15. **Martin, S., J. M. Casasnovas, D. E. Staunton, and T. A. Springer.** 1993. Efficient neutralization and disruption of rhinovirus by chimeric ICAM-1/immunoglobulin molecules. J. Virol. **67**:3561–3568.
16. **Martin, S., A. Martin, D. E. Staunton, and T. A. Springer.** 1993. Functional studies of truncated soluble ICAM-1 expressed in *Escherichia coli*. Antimicrob. Agents Chemother. **37**:1278–1284.
17. **McClelland, A., J. DeBear, S. C. Yost, A. M. Meyer, C. W. Marlor, and J. M. Greve.** 1991. Identification of monoclonal antibody epitopes and critical residues for rhinovirus binding in domain 1 of intercellular adhesion molecule 1. Proc. Natl. Acad. Sci. USA **88**:7993–7997.
18. **Ockenhouse, C. F., R. Betageri, T. A. Springer, and D. E. Staunton.** 1992. Plasmodium falciparum-infected erythrocytes bind ICAM-1 at a site distinct from LFA-1, Mac-1, and human rhinovirus. Cell **68**:63–69.
19. **Olson, N. H., P. R. Kolatkar, M. A. Oliveira, R. H. Cheng, J. M. Greve, A. McClelland, T. S. Baker, and M. G. Rossmann.** 1993. Structure of human rhinovirus complexed with its receptor molecule. Proc. Natl. Acad. Sci. USA **90**:507–511.
20. **Register, R. B., C. R. Uncapher, A. M. Naylor, D. W. Lineberger, and R. J. Colonno.** 1991. Human-murine chimeras of ICAM-1 identify amino acid residues critical for rhinovirus and antibody binding. J. Virol. **65**:6589–6596.
21. **Springer, T. A.** 1990. Adhesion receptors of the immune system. Nature **346**: 425–433.
22. **Stanley, P.** 1989. Chinese hamster ovary cell mutants with multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity. Mol. Cell. Biol. **9**:377–383.
23. **Staunton, D. E., M. L. Dustin, H. P. Erickson, and T. A. Springer.** 1990. The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. Cell **61**:243–254.
24. **Staunton, D. E., S. D. Marlin, C. Stratowa, M. L. Dustin, and T. A. Springer.** 1988. Primary structure of intercellular adhesion molecule 1 (ICAM-1) demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell **52**:925–933.
25. **Staunton, D. E., V. J. Merluzzi, R. Rothlein, R. Barton, S. D. Marlin, and T. A. Springer.** 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. Cell **56**:849–853.
26. **Tomassini, J. E., D. Graham, C. M. DeWitt, D. W. Lineberger, J. A. Rodkey, and R. J. Colonno.** 1989. cDNA cloning reveals that the major group rhinovirus receptor on HeLa cells is intercellular adhesion molecule 1. Proc. Natl. Acad. Sci. USA **86**:4907–4911.